AD_____

Award Number: DAMD17-01-1-0150

TITLE: The Non-Classical Secretion of Thioredoxin from Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Steven L. Chuck, M.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, Massachusetts 02215

REPORT DATE: June 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2003	Annual (1 Jun 02 - 31 May 03)

4. TITLE AND SUBTITLE
The Non-Classical Secretion of Thioredoxin from Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-01-1-0150

6. AUTHOR(S)
Steven L. Chuck, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Beth Israel Deaconess Medical Center
Boston, Massachusetts 02215

E-Mail: schuck@caregroup.harvard.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
Thioredoxin is a small protein with a catalytic site for redox reactions that is involved in several physiologic and pathophysiologic processes in humans. A ubiquitous cytosolic protein, thioredoxin also is secreted by some types of cells including cells derived from leukemia, lung, colon, and breast cancers. Extracellular thioredoxin acts as an autocrine growth factor and can potentiate the action of other cytokines on these tumor cell lines. The novel pathway by which thioredoxin is secreted has not been elucidated. Our specific aims are to: identify the amino acid sequence within thioredoxin that directs its secretion from cells, and to identify the cellular gene products that secrete thioredoxin from breast cancer cells. We aim to develop a molecular view of the non-classical process by which thioredoxin is secreted. Armed with a thorough understanding of this export process, it may be possible to slow the growth of breast cancers in humans in an entirely new way by inhibiting the release of thioredoxin.

14. SUBJECT TERMS
Non-classical protein secretion, thioredoxin

15. NUMBER OF PAGES
26

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Thioredoxin is a small protein with a catalytic site for redox reactions [1] that is involved in several physiologic [2-5] and pathophysiologic [6-9] processes in humans. A ubiquitous cytosolic protein, thioredoxin also is secreted by some types of cells including cells derived from leukemia, lung, colon, and breast cancers [10]. Extracellular thioredoxin acts as an autocrine growth factor and can potentiate the action of other cytokines on these tumor cell lines. In MCF-7 breast cancer cells, extracellular thioredoxin stimulates more rapid growth of the cells in culture and greater colony formation in soft agar. Thioredoxin is not exported by the classical secretory pathway involving the endoplasmic reticulum, the Golgi apparatus, and vesicular transport, nor is the protein exported via multi-drug resistance channel proteins. The novel pathway by which thioredoxin is secreted has not been elucidated. Furthermore, it is not known what the structural features of thioredoxin are that direct it but not other cytosolic proteins to a non-classical secretory pathway. Proteins secreted through the classical pathway usually contain amino-terminal signal sequences of roughly 20-30 amino acids that target the nascent chain to the membrane of the endoplasmic reticulum (ER). These sequences open the protein-conducting channel in the membrane. Similarly, nuclear, mitochondrial, and chloroplast proteins are all directed by signals encoded within the preproteins. We hypothesize that distinct amino acids in thioredoxin direct its export through a non-classical secretory pathway comprised of specific cellular proteins. Our specific aims are to:

1. Identify the amino acid sequence within thioredoxin that directs its secretion from cells.
2. Identify and clone the cellular gene products that secrete thioredoxin from breast cancer cells.

We aim to develop a molecular view of the non-classical process by which thioredoxin is secreted. Armed with a thorough understanding of this export process, it may be possible to slow the growth of breast cancers in humans in an entirely new way by inhibiting the release of thioredoxin.

Body of Report

Aim 1. Identify the amino acid sequence within thioredoxin that directs its secretion from cells

Our initial step was to establish a heterologous cell system to assay secretion of thioredoxin. This step is necessary to test the secretion of thioredoxin constructs without background signal from endogenous thioredoxin. Since thioredoxin is a ubiquitous protein, we chose a non-human cell line to assay secretion of human thioredoxin constructs. We used Chinese hamster ovary (CHO) cells because the endogenous thioredoxin is not cross-reactive with antibody against human thioredoxin, the cells are readily transfected, and secretion proved adequate.

Our plan was first to assay the secretion of truncated forms of thioredoxin to localize the region that directs secretion of the protein. Second, we planned to construct fusion proteins that could be secreted non-classically in which thioredoxin is fused in-frame with a normally cytosolic protein. We had planned to fuse short lengths of thioredoxin that are candidates for a targeting signal to this passenger and then assay for secretion.

However, we found that truncations of thioredoxin are often unstable and become degraded. Using PCR, we engineered coding regions for truncated thioredoxin (Trx) proteins corresponding to Trx91, Trx85, Trx70, and Trx55 (the number denotes the number of amino-terminal amino acids in the construct—i.e., these are all carboxy-terminal truncations). When expressed CHO cells via transient transfection using Lipofectamine 2000, these C-terminal truncations were synthesized but not secreted and instead they were degraded intracellularly over a 6-hour chase period. Therefore, we also assayed the secretion of these truncated thioredoxin proteins in the presence of protease inhibitors in an effort to prevent their intracellular degradation. First, we used acetyl-leucyl-leucyl-norleucinal (ALLN), an inhibitor of calpain proteases and proteasomes. However, we did not find significant protection of the truncated proteins nor any increase in their secretion. Second, we treated transfected cells with lactacystin, a specific and potent inhibitor of cytosolic proteasomes. Once again, however, we did not detect any secretion nor demonstrable protection from degradation. Thus, it appears that truncated thioredoxin proteins are not a suitable means of investigating a targeting sequence.

As detailed in last year's report, we have engineered and studied the secretion of several fusion proteins consisting of thioredoxin and a passenger domain. The ideal passenger domain will be long enough to permit detection by SDS-PAGE when fused to short sequences of thioredoxin. In addition, the passenger domain must be inert for non-classical secretion; that is, it must not be secreted non-classically on its own yet must be permissive for export when targeted to this pathway. The passenger domain has ranged from as short as an epitope tag (e.g., FLAG or myc) to whole proteins including green fluorescent protein (GFP)[11], glutathione S-transferase, a domain from beta lactamase, and a modified region from the FK506-binding protein. As described in last year's report, the FLAG epitope is permissive for secretion but the other passenger domains are either not secreted or not recognized by antibodies or other binding moieties. Furthermore, we found that GFP can be secreted non-classically by itself as described in last year's report [11].

Since the last report, we have investigated two other fusion proteins. First, we studied the secretion of murine dihydrofolate reductase (DHFR) and a FLAG epitope fused downstream of thioredoxin (Trx-DHFR-FLAG). This fusion protein is secreted from transfected CHO cells but only faintly detected in the medium when antibody against FLAG is used for immunoprecipitation (data not shown). We could not easily detect the FLAG epitope by western blotting either. Second, we assessed secretion of thioredoxin followed by a chitin binding domain (CBD) from *B. circulans* and the FLAG epitope (Trx-CBD-FLAG). The CBD is derived from the IMPACT intein-mediated protein expression system (New England BioLabs). This small (~6 kDa) domain has the added benefit of binding chitin beads. Hence, proper folding of this domain is assayable by binding to chitin beads. We find that Trx-CBD-FLAG is secreted from CHO cells but,

similar to other non-classically secreted thioredoxin fusion proteins and GFP by itself, it is not properly folded in the media. This conclusion is based on the binding of this fusion protein to chitin beads in cell lysates but not media. We could not detect the flag epitope well by western blotting either.

Despite these setbacks, we have examined some specific parts of thioredoxin for roles in its secretion. We engineered mutations in the coding region for thioredoxin and assessed the secretion of the expressed proteins in transfected CHO cells [12]. We found that when the redox active site of thioredoxin was rendered inactive by mutation, secretion of the protein was unaffected [12]. Similarly, mutation of the cysteine involved in the formation of homodimers did not affect secretion. Thus, these two parts of thioredoxin do not play a role in the targeting and secretion of the protein.

Our plan is to continue to assay mutations in thioredoxin for their effect on secretion of the protein. This approach will undoubtedly be more laborious than using deletions because many more mutants will need to be assessed. Since truncated thioredoxin molecules are unstable and fusion proteins have presented problems, however, this approach appears to be the next best. Using the known structure of thioredoxin, we will create specific mutations using PCR in the regions coding for externally-oriented residues in the four alpha helices and loop regions rather than the internal beta sheet regions. The mutant proteins will be assayed for secretion in CHO cells.

Aim 2. Identify and clone the cellular gene products that secrete thioredoxin from breast cancer cells

Our plan was to crosslink a bulky fusion protein that is targeted for non-classical secretion but stuck in its passage. This approach has been used successfully to crosslink components of translocation channels [13-15]. We tried using Trx-DHFR-FLAG since this construct is targeted for secretion. We treated cells expressing Trx-DHFR-FLAG with methotrexate which has been shown to bind to DHFR and induce a globular conformation. However, we found that this treatment did not impair secretion of Trx-DHFR-FLAG. Perhaps methotrexate is unable to bind to improperly-folded Trx-DHFR-FLAG molecules that are then secreted. We also examined whether there were different proteins crosslinked to a thioredoxin construct that could not be secreted but might be targeted to and "stuck" in a channel. However, we did not find reproducible differences between the crosslinking pattern using Trx-beta-lactamase, a fusion protein that is not secreted, and Trx-DHFR-FLAG, which is secreted.

We characterized in detail the secretion of thioredoxin from CHO and human tumor cells to understand the features of this secretory pathway [12]. We showed that thioredoxin is secreted non-classically from MCF-7 breast cancer cells and cells derived from other human tumors. Although secretion or surface expression of thioredoxin from these tumor lines has been shown earlier, the non-classical route had not been verified—only assumed—for any cell type other than activated lymphocytes. We demonstrated the heterologous cell system for thioredoxin secretion using transfected CHO cells. Using this system, we determined that thioredoxin is secreted slowly (over hours) but efficiently (over 50% of radiolabeled chains). We showed that low temperature affects the non-classical secretion of thioredoxin and factors in serum

inhibit its secretion. Furthermore, we showed that the mechanism of thioredoxin secretion is not the same as that of galectin-3 which is shed by membrane blebbing. Finally, we determined that the redox state of the cell does not affect the secretion of thioredoxin. Instead of blocking secretion of thioredoxin, agents such as N-acetylcysteine, which increase levels of glutathione and boost the antioxidant environment of cells, simply decrease expression of thioredoxin and thereby diminish its secretion. Our data explain the inhibitory effect of N-acetylcysteine on thioredoxin levels in patients with AIDS and in cell culture. These studies [12] more fully characterize of the non-classical secretory pathway for thioredoxin.

We investigated whether *Saccharomyces cerevisiae* are a suitable model system to study the non-classical secretion of human thioredoxin. If so, our intent was to perform mutagenesis and screen for loss of secretion of thioredoxin or a thioredoxin fusion protein. The sec18 strain has a temperature-sensitive mutation that blocks classical secretion at the non-permissive temperature. Therefore, all proteins that exit the cell at the non-permissive temperature must be secreted non-classically by definition. We transformed the sec18 strain with human thioredoxin. We detected expression of human thioredoxin in several clones of transformants. However, none of the transformants secrete thioredoxin freely into the medium. Many yeast proteins are secreted but are not liberated from beneath the rigid cell wall. We plan to try to strip the cell wall from yeast and assay for secretion of thioredoxin. We also are investigating other possible genetic systems for studying thioredoxin secretion.

Key Research Accomplishments

* Tested several thioredoxin fusion proteins for targeting and secretion

* Discovered and characterized the non-classical export of improperly-folded green fluorescent protein

* Determined that improperly-folded thioredoxin fusion proteins can be substrates for non-classical secretion

* Determined that the redox active site and dimerization site do not play roles in the targeting and secretion of thioredoxin

* Demonstrated unequivocally that thioredoxin is secreted non-classically from breast cancer cells

* Characterized in detail the non-classical secretion of thioredoxin

Reportable Outcomes

1. Tanudji M, Hevi S, and Chuck SL, *Improperly folded green fluorescent protein is secreted via a non-classical pathway.* J Cell Sci, 2002. **115**: p. 3849-57.

2. Tanudji M, Hevi S, and Chuck SL, *The nonclassic secretion of thioredoxin is not sensitive to redox state.* Am J Physiol Cell Physiol, 2003. **284**: p. C1272-9.

Conclusions

The non-classical secretion of thioredoxin from breast cancer cells has been associated with enhanced tumorigenesis. We have studied the non-classical secretion of several thioredoxin fusion proteins with the aim of defining the targeting signal and export machinery for this pathway. At present, our knowledge of this pathway cannot be developed as a medical product. We are continuing our studies, however, with the goal of completing our aims.

References

1. Holmgren A, *Redox regulation by thioredoxin and thioredoxin reductase.* Biofactors, 2000. **11**(1-2): p. 63-4.
2. Hirota K, Murata M, Sachi Y, Nakamura H, Takeuchi J, Mori K, and Yodoi J, *Distinct roles of thioredoxin in the cytoplasm and in the nucleus. A two-step mechanism of redox regulation of transcription factor NF- kappaB.* J Biol Chem, 1999. **274**(39): p. 27891-7.
3. Saitoh M, Nishitoh H, Fujii M, Takeda K, Tobiume K, Sawada Y, Kawabata M, Miyazono K, and Ichijo H, *Mammalian thioredoxin is a direct inhibitor of apoptosis signal- regulating kinase (ASK) 1.* Embo J, 1998. **17**(9): p. 2596-606.
4. Balcewicz-Sablinska MK, Wollman EE, Gorti R, and Silberstein DS, *Human eosinophil cytotoxicity-enhancing factor. II. Multiple forms synthesized by U937 cells and their relationship to thioredoxin/adult T cell leukemia-derived factor.* J Immunol, 1991. **147**(7): p. 2170-4.
5. Di Trapani G, Orosco C, Perkins A, and Clarke F, *Isolation from human placental extracts of a preparation possessing 'early pregnancy factor' activity and identification of the polypeptide components.* Hum Reprod, 1991. **6**(3): p. 450-7.
6. Tagaya Y, Okada M, Sugie K, Kasahara T, Kondo N, Hamuro J, Matsushima K, Dinarello CA, and Yodoi J, *IL-2 receptor(p55)/Tac-inducing factor. Purification and characterization of adult T cell leukemia-derived factor.* J Immunol, 1988. **140**(8): p. 2614-20.
7. Wakasugi N, Tagaya Y, Wakasugi H, Mitsui A, Maeda M, Yodoi J, and Tursz T, *Adult T-cell leukemia-derived factor/thioredoxin, produced by both human T-lymphotropic virus type I- and Epstein-Barr virus-transformed lymphocytes, acts as an autocrine growth factor and synergizes with interleukin 1 and interleukin 2.* Proc Natl Acad Sci U S A, 1990. **87**(21): p. 8282-6.
8. Wang J, Kobayashi M, Sakurada K, Imamura M, Moriuchi T, and Hosokawa M, *Possible roles of an adult T-cell leukemia (ATL)-derived factor/thioredoxin in the drug resistance of ATL to adriamycin.* Blood, 1997. **89**(7): p. 2480-7.
9. Gallegos A, Gasdaska JR, Taylor CW, Painemurrieta GD, Goodman D, Gasdaska PY, Berggren M, Briehl MM, and Powis G, *Transfection With Human Thioredoxin Increases Cell Proliferation and a Dominant-Negative Mutant*

Thioredoxin Reverses the Transformed Phenotype of Human Breast Cancer Cells. Cancer Research, 1996. **56**(24): p. 5765-5770.

10. Rubartelli A, Bajetto A, Allavena G, Wollman E, and Sitia R, *Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway.* J Biol Chem, 1992. **267**(34): p. 24161-4.
11. Tanudji M, Hevi S, and Chuck SL, *Improperly folded green fluorescent protein is secreted via a non-classical pathway.* J Cell Sci, 2002. **115**(Pt 19): p. 3849-57.
12. Tanudji M, Hevi S, and Chuck SL, *The nonclassic secretion of thioredoxin is not sensitive to redox state.* Am J Physiol Cell Physiol, 2003. **284**(5): p. C1272-9.
13. Mothes W, Prehn S, and Rapoport TA, *Systematic probing of the environment of a translocating secretory protein during translocation through the ER membrane.* Embo J, 1994. **13**(17): p. 3973-82.
14. Kouranov A and Schnell DJ, *Analysis of the interactions of preproteins with the import machinery over the course of protein import into chloroplasts.* J Cell Biol, 1997. **139**(7): p. 1677-85.
15. Eilers M and Schatz G, *Binding of a specific ligand inhibits import of a purified precursor protein into mitochondria.* Nature, 1986. **322**: p. 228-232.

Appendix

1. Tanudji M, Hevi S, and Chuck SL, *Improperly folded green fluorescent protein is secreted via a non-classical pathway.* J Cell Sci, 2002. **115**: p. 3849-57.

2. Tanudji M, Hevi S, and Chuck SL, *The nonclassic secretion of thioredoxin is not sensitive to redox state.* Am J Physiol Cell Physiol, 2003. **284**: p. C1272-9.

Improperly folded green fluorescent protein is secreted via a non-classical pathway

Marcel Tanudji, Sarah Hevi and Steven L. Chuck*

Molecular Medicine Unit, Department of Medicine, Beth Israel, Deaconess Medical Center and Harvard Medical School, Boston, MA 02215, USA

*Author for correspondence (e-mail: schuck@caregroup.harvard.edu)

Accepted 7 July 2002
Journal of Cell Science 115, 3849-3857 © 2002 The Company of Biologists Ltd
doi:10.1242/jcs.00047

Summary

The green fluorescent protein is a cytosolic protein frequently used as a molecular tag to study protein localization in intact cells. We discovered that this protein is secreted into the medium by several but not all cell lines through a non-classical secretory pathway that is insensitive to brefeldin A. Green fluorescent protein is secreted efficiently by Chinese hamster ovary cells, with 60% of synthesized proteins secreted over 8 hours. This pathway is sensitive to changes in temperature but not to factors in serum or chemicals known to affect other non-classical protein secretion pathways. Fluorescence is observed in cells expressing green fluorescent protein, indicating that some of the protein must be fully folded in the cytosol. However, secreted green fluorescent protein is not fluorescent and therefore not folded properly. Furthermore, cellular fluorescence does not change over 6 hours whereas a significant proportion of green fluorescent protein is secreted. Thus, nascent green fluorescent protein either is folded correctly or incorrectly, and the improperly folded molecules can be exported. Non-classical secretion might be a route by which cells remove an excess of improperly folded, cytosolic proteins.

Key words: Green fluorescent protein, Brefeldin A, Non-classical protein secretion, Protein trafficking

Introduction

Green fluorescent protein (GFP) provides bioluminescence in coelenterates such as the jellyfish *Aequorea victoria*. GFP folds into a barrel-shaped structure made up of beta-strands, with the chromophore located in the center (Tsien, 1998). Fully folded GFP does not require cofactors or substrate to be functional, and excitation of the chromophore at a specific wavelength results in the emission of fluorescent light (Chalfie, 1995). Furthermore, the fluorescence is stable, species-independent and can be observed non-invasively in any cell expressing GFP (Kain et al., 1995). These convenient properties of GFP have been exploited as a tool to study various cellular processes (Cinelli et al., 2000).

Owing to its intrinsic fluorescence, GFP is commonly used as a molecular tag to study intracellular protein trafficking. GFP appears to be an inert and stable molecule localized in the cytosol. Furthermore, it is small enough (29 kDa) to be used as a passenger protein for fusion constructs. In these fusion proteins, it is assumed that GFP does not contain intrinsic targeting information. To date, various proteins have been tagged with GFP to study localization and sorting in compartments such as the mitochondria, nucleus, chloroplasts, endoplasmic reticulum (ER), Golgi apparatus and plasma membrane (Chatterjee and Stochaj, 1996; Choy et al., 1999; Lee et al., 2001; Niwa et al., 1999). GFP-tagged proteins can be observed and their movement tracked in intact cells simply by looking for fluorescence at the targeted location.

Protein secretion in mammalian cells generally occurs via the classical secretory pathway that traverses the ER and Golgi apparatus. Secreted proteins contain a signal sequence with all the necessary information required to target them for secretion. A protein that is not normally secreted can be targeted for secretion by attaching a signal sequence (Simon et al., 1987). The classical secretory pathway is completely inhibited by brefeldin A (BFA), which causes reversible resorption of the Golgi apparatus back into the ER (Doms et al., 1989).

Over the past decade, it has been shown that several proteins are secreted independently of the ER-Golgi pathway. For example, basic fibroblast growth factor (FGF), interleukin (IL)-1β, HIV-tat, galectin-3, and thioredoxin are secreted in a non-classical manner (Chang et al., 1997; Mehul and Hughes, 1997; Mignatti et al., 1992; Rubartelli et al., 1992). These proteins do not display any signal sequence or protein motif known to act as a signal for export (for a review, see Muesch et al., 1990; Rubartelli and Sitia, 1997). Furthermore, their secretion is not inhibited by the addition of BFA. The pathways used for the export of these proteins are still poorly understood, and multiple pathways for non-classical protein secretion may exist in cells.

In our laboratory, we wished to use GFP as a passenger in a fusion construct to study non-classical secretion by Chinese hamster ovary (CHO) cells. As a control, we expressed GFP alone. To our surprise, most of the GFP expressed in CHO cells is secreted into the medium in a non-classical manner. Many but not all types of cells examined also secrete GFP. We characterized the kinetics, sensitivity to temperature and factors in serum and effects of known specific chemical

inhibitors on the export of GFP from transfected CHO cells. In contrast to cytosolic GFP, secreted GFP does not fluoresce, indicating that it is not properly folded.

Materials and Methods

Reagents

Trans [^{35}S]-label (>70% L-methionine; >1000 Ci/mmol) was purchased from ICN Biomedicals. Mouse monoclonal antibodies specific for GFP used for immunoprecipitation and western blotting were purchased from QBiogene. Monoclonal antibodies against the myc epitope were purified by protein G affinity chromatography from media of MYC 1-9E10.2 cells (Evan et al., 1984). Polyclonal antibodies against the FLAG epitope (DYKDDDDK) were purchased from ProSci. Protein A-agarose, Lipofectamine 2000, OptiMEM, fetal calf serum and Glutamax I were purchased from Gibco/Life Technologies. The pQBI25-fN3 plasmid, encoding modified GFP with a single emission (474 nm) and excitation peak (509 nm), is from QBiogene. BFA and cycloheximide was purchased from Sigma.

Plasmid constructions

The GFP construct used in this study was created as follows. Using the pQBI25-fN3 plasmid as a template, the forward (encoding a *Bam*HI site, translation initiation consensus sequence and initiation methionine: CGGGATCCGCCACCATGGCTAGCAAAGGAGAAG-AACTCTTC) and reverse (encoding a stop codon and *Xba*I site: CGTCTAGATAGTCAATCGATGTTGTAGAG) primers were used to amplify the GFP-coding region with Platinum pfx DNA polymerase (Gibco/ Life Technologies). The PCR product was digested, isolated and subcloned into pcDNA3 at the *Bam*HI and *Xba*I site of the multiple cloning site. A similar method was used to create the preprolactin-myc construct using the forward (CGGGATCCGCCAC-CATGGACAGCAAAGGTTCGTCGC) and reverse (ATAGTTTAG-CGGCCGCGCAGTTGTTGTTGTAGATGATTCTGC) primers to amplify the bovine preprolactin gene from the plasmid pT7-Bprl. The PCR product was isolated and cloned between the *Bam*HI and *Not*I sites of pcDNA3 with a pre-existing myc epitope coding sequence following the *Not*I site. The murine dihydrofolate reductase (DHFR)-FLAG mammalian expression construct was created as follows. Using pDS5/3 plasmid [(Rassow et al., 1989) a kind gift of Elzbieta Glaser] as a template, the DHFR gene was amplified using forward (CGGGATCCGCCACCATGGAATTCATGGTTCGACCATTG) and reverse (CCTCTAGATTACTTGTCGTCATCGTCCTTGTAGTCGT-CTTTCTTCTCGTAGACTTCAAAC) primers. The PCR product was digested with *Bam*HI and *Not*I and subcloned into pcDNA3 with a pre-existing FLAG-epitope-coding sequence following the *Not*I site. The same approach was used to create the *Schistosoma japonicum* glutathione S-transferase mammalian expression construct except that pGEX-3 plasmid (Pharmacia) was used as a template and the different forward (GGAATTCTATGTCCCCTATACTAGGTTATTGG) and reverse primers (CCTCTAGATCACGATAAATTCCGGGGATCCC) were used for amplification. All constructs were verified by DNA sequencing.

Cell culture and transient transfection

CHO, A375, COS, NIH 3T3, HEK-293, MCF-7 and HT-29 cells were maintained at 37°C/5% CO2 in culture medium (DMEM, 10% fetal bovine serum, 1% penicillin/streptomycin and 1% non-essential amino acids). One day prior to transfection, the cells were trypsinized and counted. One million cells were used to seed 60 mm culture dishes and left overnight to form >90% confluent monolayers. Confluent layers of cells were transiently transfected with Lipofectamine 2000 according to the manufacturer's instructions. Briefly, 3 μg of plasmid DNA and 15 μl of Lipofectamine 2000 were used for each transfection. Each of the components were resuspended in 450 μl of OPTI-MEM, incubated for 5 minutes at room temperature, mixed and incubated for a further 23 minutes before the addition of 4 ml of OPTI-MEM. The DNA-Lipofectamine 2000 suspension was then added to the cells (prewashed twice in PBS to remove residual serum proteins) and incubated at 37°C overnight. The next day, transfected cells were washed twice in PBS to remove residual DNA-Lipofectamine 2000 complexes prior to metabolic labeling.

Metabolic labeling with [^{35}S]-methionine

The cells were incubated in 1 ml starving medium (DMEM minus methionine and cysteine, 5% fetal bovine serum, 1% Glutamax I, 1% non-essential amino acids, 1× penicillin and streptomycin) for 30 minutes at 37°C in 5% CO_2. Metabolic labeling was carried out by the addition of 100 μCi of [^{35}S]-methionine and incubating at 37°C for 30 minutes. At the end of the labeling period, the cells were washed in 1 ml of chase medium (DMEM, 1× penicillin and streptomycin, 1% non-essential amino acids) and chased in 800 μl of chase medium for the indicated amount of time. Some cells were treated with 1 μg/ml BFA during the starvation, labeling and chase periods.

Immunoprecipitation, SDS-PAGE and phosphor-imaging

For each pulse-chase assay, the medium was collected and cells on the dish were lyzed by the addition of 1 ml of 1X Triton X-100 salt wash buffer (TXSWB; 1% Triton X-100, 100 mM Tris-HCl pH 8, 100 mM NaCl, 10 mM EDTA) in the presence of 2 mM PMSF. Both the cell lysate and medium were centrifuged at 16,000 ***g*** for 15 minutes to remove cell debris, and the supernatant was transferred to a fresh 1.5 ml tube. At this point, cleared cell lysate and medium were used for spectrofluorometric measurements or LDH assays (see below). For immunoprecipitation, 200 μl of 5× TXSWB (5% Triton X-100, 500 mM Tris-HCl pH 8, 500 mM NaCl, 50 mM EDTA) and 2 mM PMSF were added to the cleared medium. Either 1 μl of anti-GFP antibodies or 6 μl of anti-myc antibodies bound to protein G beads was added to each 1 ml of the cell lysate or medium. The samples were mixed by inversion and incubated at 4°C for 1 hour before addition of 10 μl of a suspension of protein G-agarose beads. The samples were rotated at 4°C overnight, washed twice in 1× TXSWB and twice in wash buffer (100 mM Tris-HCl pH8, 100 mM NaCl) and resuspended in 1× SDS-PAGE buffer with 500 mM DTT. The samples were incubated at 37°C for 30 minutes prior to boiling and loaded on a 15% polyacrylamide gel. At the completion of electrophoresis, the gels were destained for 30 minutes, soaked in 1 M sodium salicylate for 30 minutes and dried. The gels were exposed to x-ray film for viewing or a phosphor-imaging screen (Molecular Dynamics) for quantification.

Lactate dehydrogenase assay

Assays for lactate dehydrogenase (LDH) were carried out on 5 μl samples of cell lysate and medium after the chase period to assess cell lysis. The TOX7 LDH assay kit (Sigma) was used according to the manufacturer's instructions.

Spectrofluorometric measurements

200 μl of cleared cell lysate and medium were aliquotted into 96-well plates with blank 1× TXSWB and fresh chase medium as the respective controls. The plates were scanned on a Cytofluorescent 2350™ Fluorescence Measurement System (Millipore) with filter sets covering the GFP excitation and emission wavelengths (exciation: 485±20 nm; emission: 530±25 nm).

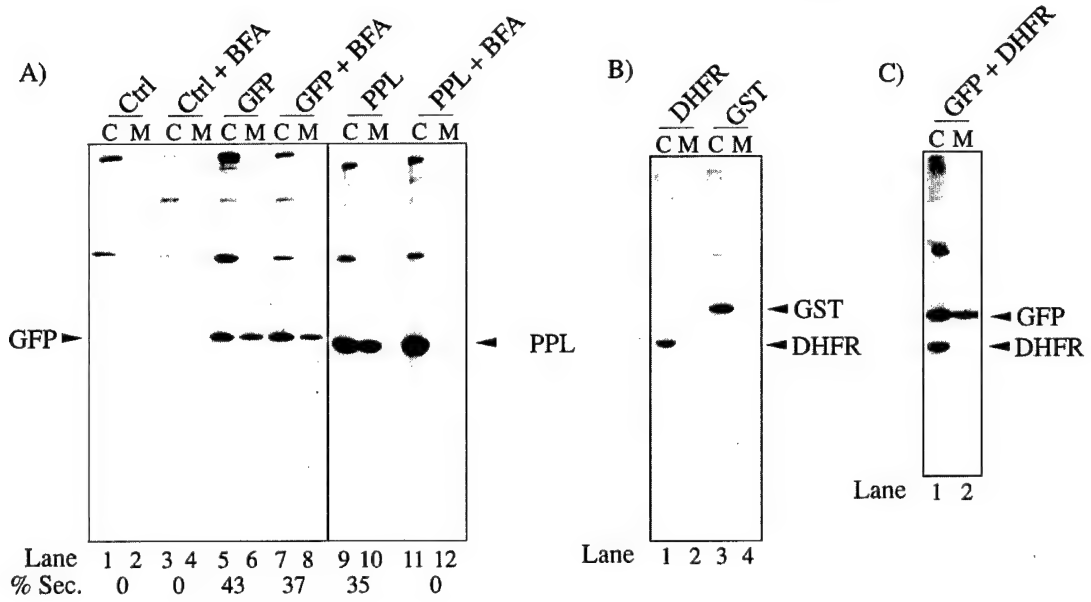

Fig. 1. GFP is secreted via a non-classical pathway in CHO cells. (A) Untransfected CHO cells (Ctrl) and cells transiently transfected with either GFP (GFP) or preprolactin-myc (PPL) were labeled with [^{35}S]-methionine and chased for 6 hours. The cell lysates (C) and media (M) were subjected to immunoprecipitation with either antibodies against GFP (lanes 1-8) or myc (lanes 9-12), and the washed immunoprecipitates were displayed by SDS-PAGE and fluorography. Some cells (+BFA) were treated with 1 μg/ml brefeldin A for 1 hour prior to labeling (lanes 3-4, 7-8 and 11-12). '% Sec.' denotes the percentage of secretion of GFP into the media as determined by a phosphorimager. (B) CHO cells transfected with plasmids encoding either mouse dihydrofolate reductase (mDHFR) or *Schistosoma japonicum* glutathione S-transferase (GST) both tagged with the FLAG epitope. The cells were labeled with [^{35}S]-methionine and chased in serum-free medium. The cell lysate (C) and media (M) were immunoprecipitated with anti-FLAG antibodies and processed as above. (C) Plasmids coding for mDHFR and GFP were used to co-transfect CHO cells. The cells were labeled, chased and processed as described in B.

Results

Transiently transfected CHO cells secrete GFP via a non-classical mechanism

We wished to use GFP as a passenger protein to assay for non-classical secretion. As a control, we expressed GFP to investigate its cellular location in CHO cells. [^{35}S]-methionine-labeled GFP was detected as an immunoreactive 29 kDa protein in cell lysate (Fig. 1, lane 5), which is absent in the lysate or medium of untransfected cells (Fig. 1A, lanes 1 and 2). To our surprise, however, we saw GFP in the medium after 6 hours of chase (Fig. 1A, lane 6). We investigated whether GFP was secreted via the classical secretory pathway. CHO cells were transiently transfected with a plasmid encoding GFP then pulse labeled and chased in the presence of 1 μg/ml BFA, an effective inhibitor of the classical secretory pathway. GFP was detected in similar amounts in both the cell lysate and medium in the absence or presence of BFA (Fig. 1A, lanes 7 and 8 versus lanes 5 and 6). To verify the effectiveness of BFA on the classical ER-Golgi pathway, CHO cells were transfected with bovine preprolactin. This protein was synthesized and secreted (Fig. 1, lanes 9 and 10), and its export was inhibited by 1 μg/ml of BFA (Fig. 1A, lanes 11 and 12). Thus, this concentration of BFA was sufficient to inhibit ER-Golgi dependent protein secretion. We confirmed that the GFP detected in the medium was secreted and not released by cell lysis by carrying out an assay for LDH. In each sample, less than 5% of total cellular LDH was detected after a 6 hour chase period (data not shown). To investigate whether other cytosolic proteins of a similar size are non-specifically exported after transient transfection, we transfected CHO cells with genes coding for murine dihydrofolate reductase (mDHFR) and *Schistosoma japonicum* glutathione S-transferase. However, overexpression of these proteins in CHO cells did not result in their secretion into the medium (Fig. 1B). Recent reports have shown that the expression of GFP in mammalian cells can be toxic (Liu et al., 1999). It is possible that GFP expression in CHO cells causes these cells to be more susceptible to lysis. Our assay for LDH does not discriminate between lysis in transfected or untransfected cells. Therefore, we co-transfected CHO cells with the plasmid constructs coding for GFP and mDHFR proteins. If GFP expression is toxic and results in cell lysis, then GFP and mDHFR proteins would be expected to be present in the medium in equivalent proportions after 6 hours of chase. When both proteins are co-expressed in CHO cells (Fig. 1C, lane 1), however, only GFP is detected in the medium after 6 hours of chase (Fig. 1C, lane 2). This result indicates that GFP is specifically secreted by CHO cells and is not present in the medium because of lysis of the transfected cells.

We also assessed the ability of other commonly used cell lines to secrete GFP. NIH 3T3 and HEK293 cells transiently transfected with a plasmid encoding GFP secrete the protein non-classically over 6 hours in the presence of BFA (Fig. 2, lanes 1 to 4). By contrast, COS cells secrete GFP poorly over 6 hours (Fig. 2, lane 5 vs 6). Certain cancer cells export thioredoxin via a non-classical pathway (Berggren et al., 1996). Therefore, we investigated whether thioredoxin-secreting cancer cells, such as A375, MCF-7 and HT-29, can also efficiently secrete GFP. We observed that GFP is generally secreted by these cancer cells in the presence of BFA (Fig. 2, lanes 7-10). One exception, however, are HT-29 cells, which

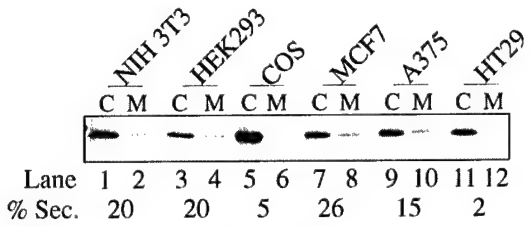

Fig. 2. GFP is secreted by various cells including cancer cells. NIH 3T3, HEK-293, COS, MCF-7, A375 and HT-29 were transiently transfected with a plasmid encoding GFP. The day after transfection, the cells were labeled with [^{35}S]-methionine and chased in the presence of BFA. After 6 hours, the cell lysates (C) and media (M) were harvested and immunoprecipitated with anti-GFP antibodies. The washed immunoprecipitates were resolved by SDS-PAGE followed by fluorography. '% Sec.' denotes the percentage of GFP secreted by transfected cells.

do not secrete GFP (Fig. 2A, lanes 11 and 12) yet export thioredoxin well (Berggren et al., 1996). For each cell line tested, no more than 5% of total cellular LDH was detected in the medium, indicating that very little cell lysis had occurred. Overall, compared with CHO cells, less GFP is secreted from the cell lines studied in Fig. 2. Taken together, these results indicate that GFP can be secreted by some cells via a brefeldin A-resistant pathway that is particularly active in CHO cells.

Kinetics of GFP secretion by transiently transfected CHO cells

We next investigated the kinetics of GFP secretion. CHO cells transfected with GFP were labeled with [^{35}S]-methionine and chased at 37°C in medium lacking serum for 2, 4, 6 and 8 hours. After separation by SDS-PAGE, the protein bands were quantified using a phosphorimager, and the relative secretion for each time point was calculated (Fig. 3). The secretion of GFP is a slow but steady process with up to 60% being secreted into the medium after 8 hours of chase. The increase in secretion is not caused by cell lysis since an assay for LDH showed that less than 5% of total cellular LDH was detected in the medium after the maximum chase time of 8 hours (data not shown). Since an adequate proportion of GFP is secreted into the medium after 6 hours of chase (Fig. 3, lanes 5 and 6), we chose to use this time point for subsequent assays of GFP secretion.

GFP secretion is altered by temperature

Facilitated protein secretion is generally a temperature-dependent process. For example, secretion of proteins via the classical ER-Golgi secretory pathway is affected by alterations in temperature (Saraste et al., 1986). On the other hand, passive diffusion through a pore is not affected by a change in temperature (Melchior and Gerace, 1995). We investigated the temperature sensitivity of GFP secretion to gain insight into whether it is a facilitated or passive process. CHO cells transfected with GFP were starved, labeled at 37°C and then incubated in serum-free chase medium at 25°C, 37°C and 42°C for 6 hours. As expected, about 45% of GFP synthesized in the cells is secreted after 6 hours at 37°C (Fig. 4, lanes 3 and 4). Lowering the chase temperature down to 25°C drastically

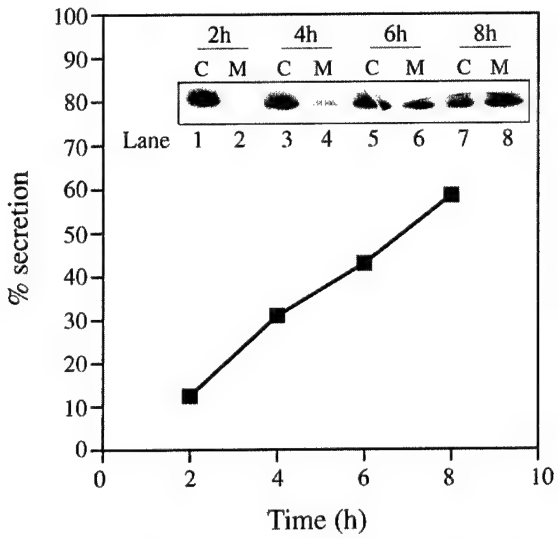

Fig. 3. Kinetics of GFP secretion from CHO cells. CHO cells transiently expressing GFP were starved, metabolically labeled with ^{35}S-methionine and chased for various times as indicated. At each time point, the cell lysate (C) and medium (M) were harvested and immunoprecipitated with anti-GFP antibodies and analyzed by SDS-PAGE followed by fluorography. The graph depicts the percentage of secretion at each time point.

inhibits GFP secretion (Fig. 4, lanes 1 and 2). On the other hand, increasing the chase temperature to 42°C increased the secretion of GFP modestly by 10% (Fig. 4, lanes 5 and 6). However, assays of LDH indicated an increase of cell lysis at this temperature (15% of total cellular LDH detected in the medium), which can account entirely for the slight increase in GFP detected in the medium.

GFP secretion is not affected by the amount of serum in the media

Some non-classical secretory pathways are sensitive to factors in serum. For example, the secretion of HIV-tat and IL-1β is inversely proportional to the amount of serum in the medium (Chang et al., 1997; Rubartelli et al., 1990). The secretion of thioredoxin is also reduced with increasing amounts of serum in the medium (M.T., S.H. and S.L.C., unpublished). We investigated whether factors present in fetal bovine serum also affect the secretion of GFP. CHO cells transfected with the GFP plasmid were starved, labeled with [^{35}S]-methionine and

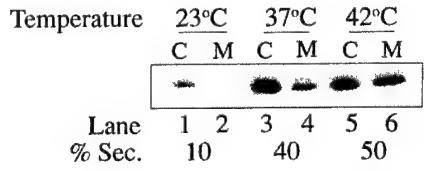

Fig. 4. GFP secretion is a temperature-dependent process. CHO cells transiently expressing GFP were starved, labeled with [^{35}S]-methionine at 37°C and chased for 6 hours at various temperatures as indicated above each set of lanes. The cell lysates (C) and media (M) were harvested and immunoprecipitated with anti-GFP antibodies. '% Sec.' denotes the percentage of secretion.

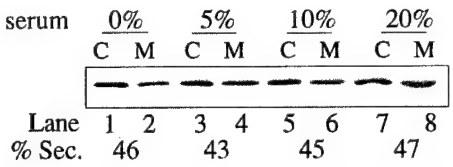

Fig. 5. GFP secretion is independent of factors present in serum. CHO cells transiently expressing GFP were starved, labeled with [^{35}S]-methionine and chased for 6 hours in the presence of various concentrations of serum as indicated. The cell lysates (C) and media (M) were harvested and immunoprecipitated with anti-GFP antibodies. '% Sec.' denotes the percentage of secretion.

chased in medium containing 0%, 5%, 10% and 20% fetal bovine serum (Fig. 5). Quantification using a phosphorimager revealed no significant differences in the proportion of secreted GFP despite increasing concentrations of serum.

Various chemicals do not affect GFP secretion

The results above suggest that GFP is secreted by a non-classical secretory pathway in CHO cells. To investigate whether the pathway for GFP secretion might be the same as that used by other non-classically secreted proteins, we tested the effect of various chemical inhibitors on the secretion of GFP (Table 1) (Hughes, 1999; Mignatti et al., 1992; Rubartelli et al., 1990). Although some of these compounds alter the non-classical secretion of other proteins, none appeared to have a significant effect on the secretion of GFP. For example, methylamine, which inhibits the non-classical secretion of thioredoxin by blast cells (Rubartelli et al., 1992), has no effect on the secretion of GFP. Compounds such as monensin and A23187, which enhance the release of thioredoxin, IL-1β and galectin-3 (Mehul and Hughes, 1997; Rubartelli and Sitia, 1991), also have no effect on GFP secretion. These data suggest that GFP may use a different non-classical secretory pathway.

Secreted GFP is not associated with externalized membrane vesicles

At least one non-classically secreted protein, galectin-3, is

Table 1. Many chemical compounds, including inhibitors of protein export, do not affect GFP secretion

Compound	Molecular target	Concentration tested
Brefeldin A	Golgi apparatus	2 μg/ml
Methylamine	Exocytosis	10 mM
Monensin	Na^+ ionophore	100 μM
Verapamil	Ca^{2+} ionophore	100 μM
Phloridzin	Glucose transporters	200 μM
Staurosporine	Protein kinase (broad)	100 nM
H-89	Protein kinase (broad)	10 μM
A23187	Ca^{2+} ionophore	1 μM
Thapsigargin	Ca^{2+} release blocker	1 μM
EDTA	Ion chelator	1 mM

CHO cells transfected with a plasmid encoding GFP were starved, labeled with [^{35}S]-methionine and chased for 6 hours in the presence of various chemicals at the concentrations indicated in the table. See text for details.

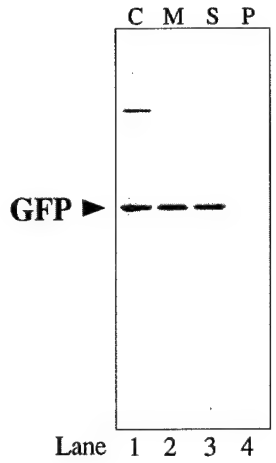

Fig. 6. Externalized membrane vesicles are not involved in GFP secretion. CHO cells transiently transfected with the plasmid encoding GFP were starved, labeled with [^{35}S]-methionine and chased for 6 hours. At the end of the chase period, the cell lysate (C) and medium (M) were collected, centrifuged at 2,000 ***g*** for 30 minutes, and each was divided into two equal aliquots. One aliquot was immunoprecipitated with anti-GFP antibodies. The second aliquot of the medium was subjected to further centrifugation at 90,000 ***g*** for 2 hours to pellet vesicles. The supernatant (S) was collected and the pellet (P) was resuspended in 1× TXSWB buffer and immunoprecipitated with anti-GFP antibodies. The samples were separated by SDS-PAGE and analyzed by autoradiography.

secreted via plasma membrane blebbing (Mehul and Hughes, 1997). We studied whether GFP is also secreted in externalized membrane vesicles. Such a mechanism could enable the post-translational export of fully folded cytosolic proteins from the cell. CHO cells transfected with the GFP plasmid were subjected to a 6 hour pulse-chase assay. At the end of the chase, the medium was clarified by low-speed centrifugation followed by high-speed centrifugation to pellet membrane vesicles in the medium (Mehul and Hughes, 1997). However, after immunoprecipitation, no GFP protein was detected in the pellet recovered after the high-speed centrifugation (Fig. 6, lane 4). Indeed, all of the GFP still remained in the supernatant fraction (Fig. 6, lane 3) and in similar amounts to that detected in the medium after low-speed centrifugation (Fig. 6, lane 2). This result suggests that GFP is not secreted into the medium via membrane blebbing.

Improperly folded GFP is secreted

GFP is a stable molecule that is resistant to spontaneous unfolding or degradation in the cell (Bokman and Ward, 1981). By fluorescence microscopy, GFP-transfected CHO cells are brightly fluorescent (data not shown) indicating that some of the GFP is folded properly in these cells. However, a loosely folded or unfolded conformation is generally required for protein translocation through channels (Schatz and Dobberstein, 1996). Since the structure of fluorescent GFP is a bulky beta barrel, it would seem likely that the protein must be at least partly unfolded for export through a channel. We investigated whether GFP is secreted as an unfolded molecule by assessing its fluorescence in the medium. First, we assessed the quantities of unlabeled GFP secreted into the media relative

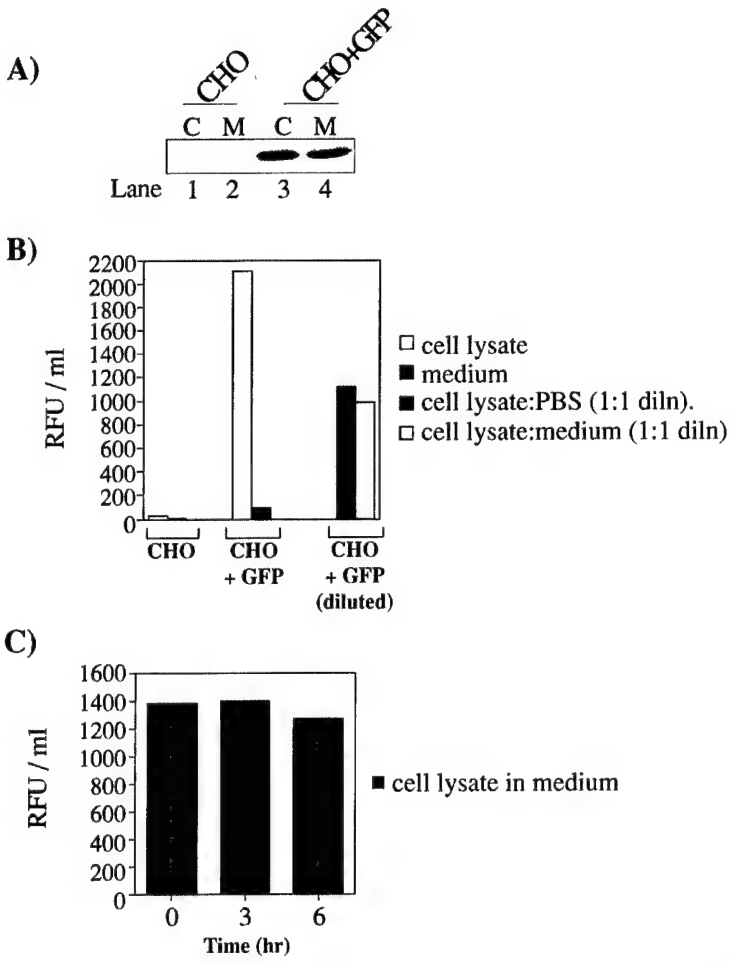

Fig. 7. Non-classically secreted GFP is not fluorescent. Untransfected (CHO) and GFP-transfected CHO cells (CHO+GFP) were washed and incubated in fresh medium for 6 hours. (A) The cell lysate (C) and media (M) were immunoprecipitated with anti-GFP antibodies, separated by SDS-PAGE and immunoblotted with anti-GFP antibodies. (B) The fluorescence was measured from 200 μl aliquots of the cell lysate and the media harvested as above. The graph shows the relative fluorescence units (RFU) per ml for the samples. The fluorescence was also measured from the lysate of GFP-expressing cells diluted 1:1 in fresh medium or PBS (CHO+GFP lysate diluted). Each bar on the graph represents the average value derived from at least three independent samples. (C) CHO cells expressing GFP were lyzed in medium supplemented with 1× Triton X-100. The fluorescence was measured from 200 μl aliquots of the medium after 0, 3 and 6 hours of incubation at 37°C. The values are plotted as RFU per ml.

to the cell by carrying out a western blot analysis. Untransfected and transfected CHO cells were placed in serum-free medium for 6 hours. At the end of the incubation, the medium and cell lysate were harvested, aliquots were saved for spectrofluorometric assays and the rest of the samples were subjected to immunoprecipitation, separated by SDS-PAGE and immunoblotted with anti-GFP antibodies (Fig. 7A). As expected, no GFP was detected in untransfected CHO cells and medium (Fig. 7A, lanes 1 and 2). GFP was detected in comparable amounts in the cells and medium of transfected cells by immunoblotting (Fig. 7A, lanes 3 and 4). We then carried out the spectrofluorometric assay on aliquots of the same samples used for the western blot. Very little auto-fluorescence was detected in the cell lysate or medium of untransfected CHO cells (Fig. 7B, CHO). On the other hand, the cell lysate of GFP-transfected CHO cells showed a large increase in fluorescence in agreement with the fluorescence seen by microscopy (Fig. 7B, CHO+GFP). However, no change in fluorescence was seen in the medium despite significant secretion of GFP (Fig. 7A).

We ruled out two possible artifacts that might account for the lack of fluorescence in the media. First, to test whether the lack of GFP fluorescence was caused by quenching by components in the medium, we carried out a 1:1 dilution of cell lysate with fresh medium or phosphate-buffered saline (PBS). The relative fluorescence detected in the sample diluted with fresh medium was half that of the undiluted sample and similar in value to the fluorescence measured when PBS was used as a diluent, indicating that the medium does not quench GFP fluorescence (Fig. 7B, CHO+GFP lysate diluted). Second, we examined whether the lack of fluorescence in the medium results from GFP becoming non-fluorescent after export into the medium. CHO cells expressing GFP were lyzed in fresh medium containing 1% Triton X-100. After clarification by centrifugation, the medium containing released cytosolic GFP was incubated at 37°C for 0, 3 and 6 hours, and the fluorescence was measured at each time point. Fluorescence remained virtually constant over 6 hours of incubation in medium (Fig. 7C). Thus, the lack of fluorescence in the medium is not caused by inactivation or unfolding of functional GFP once it is exported. From these results, we conclude that secreted GFP is not folded properly and therefore not fluorescent.

Two different forms of GFP are present in CHO cells but only the improperly folded form is secreted

The secretion of unfolded, non-fluorescent GFP could result from either of two possible models. In the first model, all cytosolic GFP is fluorescent, and to be exported it must first be unfolded prior to or during secretion. This implies that the reduction in intracellular GFP would be reflected by a similar reduction in fluorescence under conditions where no new GFP molecules are synthesized. In the second model, GFP is present in the cell in two different forms as either a properly folded, fluorescent molecule that is not secreted or as an unfolded protein that can be secreted. In this model, the fluorescence from cellular GFP would remain constant despite a decrease in the total cytosolic GFP.

We investigated these two models of GFP secretion. CHO cells transiently transfected with GFP were placed in medium supplemented with cycloheximide to a final concentration of 100 μM, which is sufficient to inhibit protein synthesis (data not shown). The cells were incubated at 37°C in this medium for 0 or 6 hours, and the media and cell lysates were harvested. Aliquots were saved for spectrofluorometric assays, and the rest of the samples were analyzed by western blotting (Fig. 8A). As expected, initially all of the GFP synthesized is present in the cell (Fig. 8A, lane 1) and not in the medium (Fig. 8A,

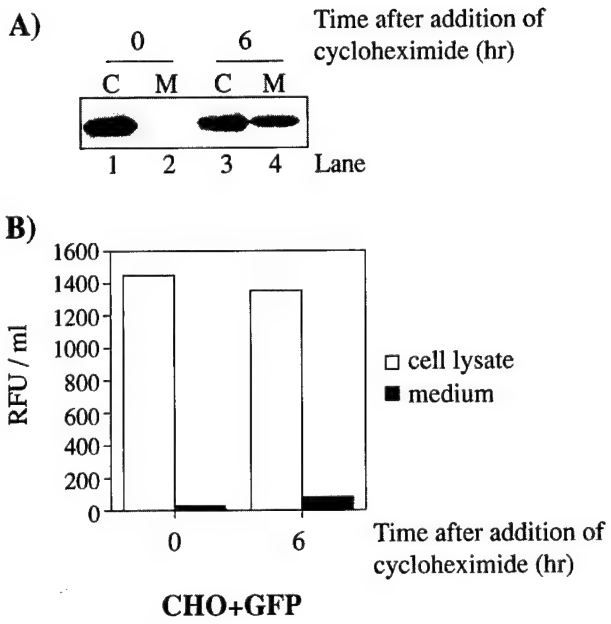

Fig. 8. Two different forms of GFP are present in CHO cells but only the improperly folded form is secreted. CHO cells expressing GFP (CHO+GFP) were incubated at 37°C in the presence of 100 μM cycloheximide for 0 or 6 hours. (A) The cell lysate (C) and media (M) were harvested at the end of the incubation, immunoprecipitated and immunoblotted with anti-GFP antibodies. (B) Spectrofluorometric measurements of 200 μl aliquots of the cell lysate and media harvested as above are plotted as RFU per ml. Each bar on the graph represents the average value derived from five independent samples.

lane 2). This data correlated with the spectrofluorometric result that showed that all of the fluorescence is associated in the cell lysate and none is detected in the medium (Fig. 8B, 0 hours chase, cell lysate versus medium). After 6 hours of chase, the immunoblot showed that approximately one third of the synthesized GFP is present in the medium (Fig. 8A, lanes 4), with a similar reduction of GFP in the cell lysate (Fig. 8A, lane 3). The relative amount of GFP secreted into the medium is similar to the secretion observed in the absence of cycloheximide (Fig. 7A), indicating that the protein synthesis inhibitor did not affect the non-classical export of GFP. Assays for LDH confirmed that the GFP dectected in the medium does not result from lysis caused by cycloheximide (data not shown). However, the fluorescence in the cell lysate after 6 hours is virtually identical to that at the outset (Fig. 8B, 6 hours, cell lysate) whereas no significant fluorescence is detected in the medium despite the significant secretion of GFP protein (Fig. 8B, 6 hours, medium). These data strongly support the second model. Thus, GFP exists in two different pools in transiently transfected CHO cells – a pool of properly folded, fluorescent molecules, and a pool of unfolded, non-fluorescent proteins – and only the second form can be secreted (Fig. 9).

Discussion

We demonstrate that GFP is secreted by a variety of cells via a non-classical secretory pathway. Significant secretion was observed from transiently transfected CHO cells with over half

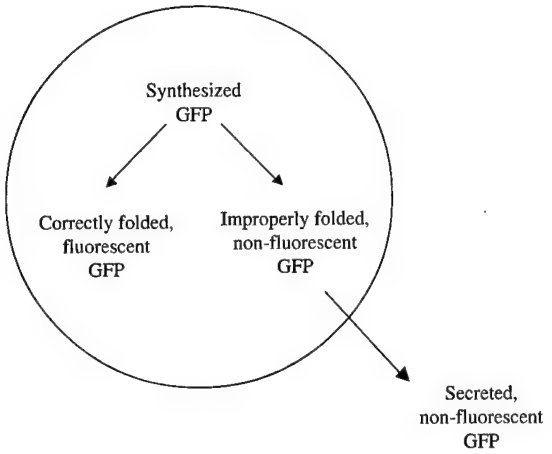

Fig. 9. The two-pool model of GFP secretion. See text for details.

of the labeled GFP exported to the medium. The secretion of GFP is sensitive to low temperature but not factors in the serum. Commonly used cell lines and several cancer cell lines also demonstrate the ability to secrete GFP albeit with less efficiency. GFP secreted from transiently transfected CHO cells does not fluoresce, indicating that the improperly folded form is secreted via this non-classical pathway.

It is surprising that GFP is secreted from transiently transfected cells. GFP is a cytosolic protein with no known targeting signal. The intrinsic targeting of GFP for non-classical export might not have been observed previously because the molecule is often targeted to a specific location in cells. To our knowledge, this is the first report of GFP secretion by mammalian cells. In yeast, GFP can be localized to the vacuole presumably through a cryptic targeting signal (Kunze et al., 1999). Previously assumed to be an inert molecule, GFP has other unanticipated effects on cells. For example, transgenic mice that express GFP in the heart develop cardiomyopathy (Huang et al., 2000). Furthermore, prolonged overexpression of GFP triggers apoptosis in cells (Liu et al., 1999). Many visual applications, such as fluorescence microscopy, may not be sensitive to minor toxic effects that go unnoticed (Schmitz and Bereiter-Hahn, 2001). Taken together, the unanticipated effects and secretion of GFP raise doubts regarding the inertness of this molecule.

Our experiments indicate that the secretion of GFP is not simply due to cell lysis caused by either a cytotoxic effect of GFP or liposome-mediated transfection (Fig. 1C). GFP but not mDHFR is secreted from CHO cells that are co-transfected with cDNA encoding both proteins. If GFP or liposome-mediated transfection caused cell lysis, both expressed proteins would be found in the media. Furthermore, CHO cells, when transfected using calcium phosphate instead of a lipsosome formulation, also secrete GFP (data not shown). Finally, only non-fluorescent GFP is secreted (Fig. 7). If released by cell lysis, both fluorescent and non-fluorescent GFP would be expected in the media. Hence, the export process can discriminate between properly and improperly folded forms. This specificity indicates that GFP is not released by cell lysis.

Although GFP can diffuse through the nuclear pore into the nucleus (Chatterjee and Stochaj, 1996; Chatterjee and Stochaj, 1998), we believe that the export of GFP is not caused by non-

selective diffusion for several reasons. First, lowering the chase temperature to 25°C significantly inhibits GFP secretion (Fig. 4). Low temperatures inhibit a variety of facilitated translocation processes but not diffusion (Melchior and Gerace, 1995). Second, GFP is poorly secreted from certain cell lines such as COS and HT-29 (Fig. 2, lanes 6 and 12). This result suggests that certain cellular factors or machinery are required for GFP export and that export of GFP is not caused by non-specific diffusion of a molten globule isoform through the plasma membrane as has been suggested for other proteins (Bychkova et al., 1988). Such translocation machinery has been characterized in the ER, mitochondria and chloroplasts (Chen et al., 2000; Johnson and van Waes, 1999; Rehling et al., 2001). Finally, the pathway used by GFP for export appears to be selective since other heterologous, cytosolic proteins overexpressed in CHO cells are not secreted (Fig. 1B,C). This selectivity implies that a specific region of the GFP protein may behave as a cryptic targeting signal as has been demonstrated in yeast (Kunze et al., 1999). Thus, the non-classical secretion of GFP is selective and not universal among mammalian cells.

Little is known about non-classical secretory pathways in eukaryotes. Several proteins such as basic FGF, IL-1β, HIV-Tat, thioredoxin and galectin-3 are secreted in a non-classical manner (Cleves, 1997; Rubartelli and Sitia, 1997). However, only a few of these pathways have been studied, and their exact mechanisms have not been identified. For example, the Na^+/K^+ ion channel has been implicated in the secretion of basic FGF (Florkiewicz et al., 1998), whereas an ATP-binding cassette (ABC) transporter appears to be involved in the export of IL-1β (Andrei et al., 1999). Galectin-3 is secreted in vesicles from membrane blebbing; this pathway appears to be capable of post-translational export of fully folded proteins (Hughes, 1999). We failed to detect any GFP associated with vesicles. Furthermore, factors in serum and various chemical inhibitors that affect the secretion of other non-classically secreted proteins have no apparent effect on GFP secretion. Our data suggest the existence of another non-classical export pathway in eukaryotic cells capable of secreting unfolded GFP.

Protein translocation across membranes generally requires proteins to be in a loosely folded or unfolded conformation. One exception is the prokaryotic twin arginine translocase (Tat), which is capable of exporting fully folded proteins – including GFP – into the periplasm (Berks et al., 2000; Thomas et al., 2001). In our studies, the GFP secreted from CHO cells is not fluorescent and therefore not properly folded. Perhaps the GFP is loosely folded or unfolded as a condition for secretion. Once secreted, GFP does not fold into a fluorescent conformation because of the absence of chaperone proteins (Feilmeier et al., 2000; Sacchetti et al., 2001). By contrast, GFP secreted via the ER-Golgi pathway is fluorescent in the medium because proper folding is maintained during secretion (Laukkanen et al., 1996).

GFP is fluorescent in the cytoplasm of transfected cells, indicating that some of the protein is properly folded. The GFP in the medium, however, is not fluorescent (Fig. 7B). Thus, to be secreted by CHO cells, GFP must be in an unfolded conformation. Two models could account for this unfolding. In the first model, fully folded GFP must be unfolded prior to or during its export. In the second model, not all of the GFP synthesized in the cytosol is folded to the fluorescent conformation. This pool of nascent, unfolded GFP might remain associated with chaperones in the cytosol to maintain a loosely folded or unfolded conformation. This scenario is much more likely for post-translationally translocated proteins (Schatz and Dobberstein, 1996). The prolonged but efficient secretion of GFP despite treatment with cycloheximide (Fig. 8) suggests that export occurs post-translationally. We discovered that the pool of intracellular, fluorescent GFP did not decrease over 6 hours, whereas a substantial fraction was secreted into the medium (Fig. 8). This result indicates that the second model is correct: two separate pools of nascent GFP – one folded and fluorescent, the other unfolded, non-fluorescent and able to be secreted – exist in cells (see model in Fig. 9). Being a protein of jellyfish origin, GFP does not always fold properly at 37°C (Ogawa et al., 1995; Patterson et al., 1997; Siemering et al., 1996; Tsien, 1998). Thus, the two pools of GFP in CHO cells probably arise from an inefficiency in proper folding even though we used an engineered form of GFP. Our data does not indicate the relative size of these two pools, although the high proportion that is secreted over several hours (Fig. 3) suggests that the pool of unfolded, non-fluorescent GFP consists of at least half the newly synthesized GFP. The pool of unfolded GFP does not appear to localize in aggresomes formed by aggregates of misfolded proteins in the cytosol (Garcia-Mata et al., 1999; Garcia-Mata et al., 2002). We observed a diffuse pattern of GFP fluorescence throughout the cytosol as is typically observed (Garcia-Mata et al., 1999; Kain et al., 1995). By immunofluorescence microscopy, an identical pattern of GFP staining is seen in the cytosol of these cells (data not shown). Thus, in cells, two overlapping pools of GFP can be found throughout the cytosol.

Since GFP is not present endogenously in CHO cells, the physiological substrate for this non-classical pathway remains to be identified. To be active extracellularly, proteins secreted via this pathway must be able to attain the proper conformation outside the cell in the absence of chaperone proteins. Alternatively, this non-classical secretory pathway might be a means to dispose of improperly folded proteins from the cytosol.

This work was supported by grant DAMD17-01-1-0150 from the United States Army.

References

Andrei, C., Dazzi, C., Lotti, L., Torrisi, M. R., Chimini, G. and Rubartelli, A. (1999). The secretory route of the leaderless protein interleukin 1beta involves exocytosis of endolysosome-related vesicles. *Mol. Biol. Cell* **10**, 1463-1475.

Berggren, M., Gallegos, A., Gasdaska, J. R., Gasdaska, P. Y., Warneke, J. and Powis, G. (1996). Thioredoxin and thioredoxin reductase gene expression in human tumors and cell lines, and the effects of serum stimulation and hypoxia. *Anticancer Res.* **16**, 3459-3466.

Berks, B. C., Sargent, F. and Palmer, T. (2000). The Tat protein export pathway. *Mol. Microbiol.* **35**, 260-274.

Bokman, S. H. and Ward, W. W. (1981). Renaturation of *Aequorea* gree- fluorescent protein. *Biochem. Biophys. Res. Commun.* **101**, 1372-1380.

Bychkova, V. E., Pain, R. H. and Ptitsyn, O. B. (1988). The 'molten globule' state is involved in the translocation of proteins across membranes? *FEBS Lett.* **238**, 231-234.

Chalfie, M. (1995). Green fluorescent protein. *Photochem. Photobiol.* **62**, 651-656.

Chang, H. C., Samaniego, F., Nair, B. C., Buonaguro, L. and Ensoli, B. (1997). HIV-1 Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulfate proteoglycans through its basic region. *Aids* **11**, 1421-1431.

Chatterjee, S. and Stochaj, U. (1996). Monitoring nuclear transport in HeLa cells using the green fluorescent protein. *Biotechniques* **21**, 62-63.

Chatterjee, S. and Stochaj, U. (1998). Diffusion of proteins across the nuclear envelope of HeLa cells. *Biotechniques* **24**, 668-674.

Chen, K., Chen, X. and Schnell, D. J. (2000). Mechanism of protein import across the chloroplast envelope. *Biochem. Soc. Trans.* **28**, 485-491.

Choy, E., Chiu, V. K., Silletti, J., Feoktistov, M., Morimoto, T., Michaelson, D., Ivanov, I. E. and Philips, M. R. (1999). Endomembrane trafficking of ras: the CAAX motif targets proteins to the ER and Golgi. *Cell* **98**, 69-80.

Cinelli, R. A., Ferrari, A., Pellegrini, V., Tyagi, M., Giacca, M. and Beltram, F. (2000). The enhanced green fluorescent protein as a tool for the analysis of protein dynamics and localization: local fluorescence study at the single-molecule level. *Photochem. Photobiol.* **71**, 771-776.

Cleves, A. E. (1997). Protein transports: the nonclassical ins and outs. *Curr. Biol.* **7**, R318-320.

Doms, R. W., Russ, G. and Yewdell, J. W. (1989). Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. *J. Cell Biol.* **109**, 61-72.

Evan, G. I., Lewis, G. K. and Bishop, J. M. (1984). Isolation of monoclonal antibodies specific for products of avian oncogene myb. *Mol. Cell. Biol.* **4**, 2843-2850.

Feilmeier, B. J., Iseminger, G., Schroeder, D., Webber, H. and Phillips, G. J. (2000). Green fluorescent protein functions as a reporter for protein localization in Escherichia coli. *J. Bacteriol.* **182**, 4068-4076.

Florkiewicz, R. Z., Anchin, J. and Baird, A. (1998). The inhibition of fibroblast growth factor-2 export by cardenolides implies a novel function for the catalytic subunit of Na^+,K^+-ATPase. *J. Biol. Chem.* **273**, 544-551.

Garcia-Mata, R., Bebok, Z., Sorscher, E. J. and Sztul, E. S. (1999). Characterization and dynamics of aggresome formation by a cytosolic GFP-chimera. *J. Cell Biol.* **146**, 1239-1254.

Garcia-Mata, R., Gao, Y. S. and Sztul, E. (2002). Hassles with taking out the garbage: aggravating aggresomes. *Traffic* **3**, 388-396.

Huang, W. Y., Aramburu, J., Douglas, P. S. and Izumo, S. (2000). Transgenic expression of green fluorescence protein can cause dilated cardiomyopathy. *Nat. Medicine* **6**, 482-483.

Hughes, R. C. (1999). Secretion of the galectin family of mammalian carbohydrate-binding proteins. *Biochim. Biophys. Acta.* **1473**, 172-185.

Johnson, A. E. and van Waes, M. A. (1999). The translocon: a dynamic gateway at the ER membrane. *Annu. Rev. Cell Dev. Biol.* **15**, 799-842.

Kain, S. R., Adams, M., Kondepudi, A., Yang, T. T., Ward, W. W. and Kitts, P. (1995). Green fluorescent protein as a reporter of gene expression and protein localization. *Biotechniques* **19**, 650-655.

Kunze, I. I., Hensel, G., Adler, K., Bernard, J., Neubohn, B., Nilsson, C., Stoltenburg, R., Kohlwein, S. D. and Kunze, G. (1999). The green fluorescent protein targets secretory proteins to the yeast vacuole. *Biochim. Biophys. Acta.* **1410**, 287-298.

Laukkanen, M. L., Oker-Blom, C. and Keinanen, K. (1996). Secretion of green fluorescent protein from recombinant baculovirus-infected insect cells. *Biochem. Biophys. Res. Commun.* **226**, 755-761.

Lee, Y. J., Kim, D. H., Kim, Y. W. and Hwang, I. (2001). Identification of a signal that distinguishes between the chloroplast outer envelope membrane and the endomembrane system in vivo. *Plant Cell* **13**, 2175-2190.

Liu, H. S., Jan, M. S., Chou, C. K., Chen, P. H. and Ke, N. J. (1999). Is green fluorescent protein toxic to the living cells?. *Biochem. Biophys. Res. Commun.* **260**, 712-717.

Mehul, B. and Hughes, R. C. (1997). Plasma membrane targetting, vesicular budding and release of galectin 3 from the cytoplasm of mammalian cells during secretion. *J. Cell Sci.* **110**, 1169-1178.

Melchior, F. and Gerace, L. (1995). Mechanisms of nuclear protein import. *Curr. Opin. Cell Biol.* **7**, 310-318.

Mignatti, P., Morimoto, T. and Rifkin, D. B. (1992). Basic fibroblast growth factor, a protein devoid of secretory signal sequence, is released by cells via a pathway independent of the endoplasmic reticulum-Golgi complex. *J. Cell Physiol.* **151**, 81-93.

Muesch, A., Hartmann, E., Rohde, K., Rubartelli, A., Sitia, R. and Rapoport, T. A. (1990). A novel pathway for secretory proteins? *Trends Biochem. Sci.* **15**, 86-88.

Niwa, Y., Hirano, T., Yoshimoto, K., Shimizu, M. and Kobayashi, H. (1999). Non-invasive quantitative detection and applications of non-toxic, S65T-type green fluorescent protein in living plants. *Plant J.* **18**, 455-463.

Ogawa, H., Inouye, S., Tsuji, F. I., Yasuda, K. and Umesono, K. (1995). Localization, trafficking, and temperature-dependence of the Aequorea green fluorescent protein in cultured vertebrate cells. *Proc. Natl. Acad. Sci. USA* **92**, 11899-11903.

Patterson, G. H., Knobel, S. M., Sharif, W. D., Kain, S. R. and Piston, D. W. (1997). Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. *Biophys. J.* **73**, 2782-2790.

Rassow, J., Guiard, B., Wienhues, U., Herzog, V., Hartl, F. U. and Neupert, W. (1989). Translocation arrest by reversible folding of a precursor protein imported into mitochondria. A means to quantitate translocation contact sites. *J. Cell Biol.* **109**, 1421-1428.

Rehling, P., Wiedemann, N., Pfanner, N. and Truscott, K. N. (2001). The mitochondrial import machinery for preproteins. *Crit. Rev. Biochem. Mol. Biol.* **36**, 291-336.

Rubartelli, A. and Sitia, R. (1991). Interleukin 1 beta and thioredoxin are secreted through a novel pathway of secretion. *Biochem. Soc. Trans.* **19**, 255-259.

Rubartelli, A. and Sitia, R. (1997). Secretion of mammalian proteins that lack a signal sequence. In *Unusual Secretory Pathways: From Bacteria to Man.* (ed. K. Kuchler, A. Rubartelli and B. Hollan), pp. 87-114. New York: R.G. Landes Company.

Rubartelli, A., Cozzolino, F., Talio, M. and Sitia, R. (1990). A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. *EMBO J.* **9**, 1503-1510.

Rubartelli, A., Bajetto, A., Allavena, G., Wollman, E. and Sitia, R. (1992). Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway. *J. Biol. Chem.* **267**, 24161-24164.

Sacchetti, A., Cappetti, V., Marra, P., Dell'Arciprete, R., El Sewedy, T., Crescenzi, C. and Alberti, S. (2001). Green Fluorescent Protein variants fold differentially in prokaryotic and eukaryotic cells. *J. Cell. Biochem.* **81**, 117-128.

Saraste, J., Palade, G. E. and Farquhar, M. G. (1986). Temperature-sensitive steps in the transport of secretory proteins through the Golgi complex in exocrine pancreatic cells. *Proc. Natl. Acad. Sci. USA* **83**, 6425-6429.

Schatz, G. and Dobberstein, B. (1996). Common principles of protein translocation across membranes. *Science* **271**, 1519-1526.

Schmitz, H. D. and Bereiter-Hahn, J. (2001). GFP associates with microfilaments in fixed cells. *Histochem. Cell Biol.* **116**, 89-94.

Siemering, K. R., Golbik, R., Sever, R. and Haseloff, J. (1996). Mutations that suppress the thermosensitivity of green fluorescent protein. *Curr. Biol.* **6**, 1653-1663.

Simon, K., Perara, E. and Lingappa, V. R. (1987). Translocation of globin fusion proteins across the endoplasmic reticulum membrane in *Xenopus laevis* oocytes. *J. Cell Biol.* **104**, 1165-1172.

Thomas, J. D., Daniel, R. A., Errington, J. and Robinson, C. (2001). Export of active green fluorescent protein to the periplasm by the twin- arginine translocase (Tat) pathway in *Escherichia coli*. *Mol. Microbiol.* **39**, 47-53.

Tsien, R. Y. (1998). The green fluorescent protein. *Annu. Rev. Biochem.* **67**, 509-544.

Am J Physiol Cell Physiol 284: C1272–C1279, 2003.
First published January 15, 2003; 10.1152/ajpcell.00521.2002.

The nonclassic secretion of thioredoxin is not sensitive to redox state

Marcel Tanudji, Sarah Hevi, and Steven L. Chuck
Molecular Medicine Unit, Department of Medicine, Beth Israel, Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215

Submitted 11 November 2002; accepted in final form 15 January 2003

Tanudji, Marcel, Sarah Hevi, and Steven L. Chuck. Nonclassic secretion of thioredoxin is not sensitive to redox state. *Am J Physiol Cell Physiol* 284: C1272–C1279, 2003. First published January 15, 2003; 10.1152/ajpcell.00521.2002.— Thioredoxin (Trx) is a cytosolic, redox-active protein that is secreted from many cells and has several extracellular functions. In activated lymphocytes, the pathway of secretion does not involve the Golgi apparatus. Levels of extracellular Trx are decreased by the antioxidant *N*-acetylcysteine. Hence, the secretion of Trx could be altered by the redox status of the cell or the protein. To study Trx mutants, we characterized the secretion of human Trx from Chinese hamster ovary cells. Secretion of human Trx is unaffected by brefeldin A, slow but efficient, and sensitive to low temperature and factors in serum. We demonstrate that *N*-acetylcysteine reduces the cellular level of Trx but not the proportion secreted; thus this chemical does not block the nonclassic pathway for Trx secretion. Furthermore, we find that mutations in either the active site or the dimerization site of Trx do not alter its secretion. Thus the nonclassic secretion of Trx is not dependent on the redox status of either the cell or the protein.

protein trafficking; protein secretion

THIOREDOXIN (Trx) is a small, redox-active, cytosolic protein found in plants, bacteria, and eukaryotic organisms that has diverse cellular functions (1, 27). In humans, a single gene for Trx encodes a protein of 105 amino acids with a molecular mass of 12 kDa that forms a compact spherical structure (13, 42, 44). Trx is an antioxidant that helps to maintain the cytosolic redox environment for proper protein folding and activates transcription factors such as necrosis factor κB (NF)-κB and activator protein-1 (AP-1) (17, 23). The active site of human Trx consists of Cys^{32}-Gly-Pro-Cys^{35}, and this motif is highly conserved among various species (1). The active site undergoes reversible oxidation-reduction, and oxidized Trx is reduced by the NADPH-dependent flavoprotein Trx reductase (43). In cancer cells, increased expression of Trx provides resistance against various anticancer drugs and protection from apoptosis (2, 4, 32).

Trx is secreted from a variety of normal and neoplastic cells of the blood, breast, colon, lung, and liver (4, 28, 30, 31, 37, 38, 40). Trx is found extracellularly despite lacking a typical, amino-terminal signal sequence that is usually present in proteins secreted via the endoplasmic reticulum (ER). Agents that disrupt secretion through the classic ER-Golgi route, such as brefeldin A (BFA) and monensin, have no effect or increase the secretion of Trx from activated lymphocytes (28). Although it is known that Trx is secreted from lymphocytes via a nonclassic pathway, this finding has not been corroborated for other cell types. The details of the secretory pathway through which Trx is exported are unknown.

Extracellular Trx performs a variety of physiological and pathophysiological functions. Trx is a powerful chemotactic factor for eosinophils as part of the defense against parasites (3, 34, 35). Trx is also a part of the early pregnancy factor necessary for establishment of the placenta (8, 9). Once secreted, Trx can potentiate the effect of cytokines such as interleukins-1 and -2 (15, 41). Furthermore, Trx on its own can stimulate growth and promote the survival of cells (15). Indeed, Trx was identified as the essential growth-promoting protein in the conditioned medium of adult T cell leukemia (ATL) cells (37, 38). The reducing activity of Trx is necessary for its mitogenic activity (14). Recent studies have shown that Trx reductase is also secreted by both normal and neoplastic cells (36). Trx reductase is therefore available to regenerate reduced Trx extracellularly.

Trx can also alter the function of lymphocytes and neutrophils (26). Exogenous Trx protects lymphoid cells against TNF-α- or hydrogen peroxide-mediated cytotoxicity and prevents apoptosis caused by depletion of glutathione and L-cysteine (18). In contrast to these beneficial effects, elevated plasma levels of Trx can block chemotaxis by neutrophils in response to lipopolysaccharide (25). An elevated level of plasma Trx was found to be associated with decreased survival in patients with acquired immune deficiency syndrome (AIDS) and correlated with reduced chemotaxis of neutrophils (24). Chemicals that are antioxidants or that increase glutathione could lower levels of Trx and thereby diminish its detrimental effects on neutrophils. *N*-acetylcysteine (NAC) is itself an antioxidant

Address for reprint requests and other correspondence: S. Chuck, Molecular Medicine Unit, Beth Israel Deaconess Medical Center, RW663, 330 Brookline Ave., Boston, MA 02215 (E-mail: schuck@bidmc.harvard.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

and also increases cellular glutathione. The administration of NAC increased survival in patients with AIDS (24).

Treatment with NAC was also reported to decrease the secretion of Trx by human T-cell leukemia virus I-transformed T cells (24). These findings raise the issue of whether NAC blocks the nonclassic secretory pathway or simply lowers cytosolic levels of Trx. We investigated this issue because no specific inhibitor of the nonclassic secretory pathway used by Trx is known. We also examined whether the secretion of Trx is sensitive to the redox status of the cell or Trx itself.

We investigated the heterologous secretion of human Trx from Chinese hamster ovary (CHO) cells. This approach enables the study of mutant forms of Trx without cross-reacting, endogenous human Trx. Similar to cancer cells that secrete endogenous Trx, transfected CHO cells secrete human Trx slowly but efficiently. This export is not inhibited by BFA, indicating that a nonclassic pathway is used. We characterize the secretion of Trx from CHO cells and demonstrate that its exportation does not depend on either the redox status of the cell or a functional active site in Trx.

METHODS

Reagents. Emmanuelle Wollman kindly provided the plasmid-encoding Trx (44), and David Silberstein generously provided polyclonal antibodies for Trx (3). Monoclonal antibodies against the myc epitope were purified by protein G affinity chromatography from media of MYC 1-9E10.2 cells (10). Lipofectamine 2,000, OPTI-MEM, Glutamax I, penicillin/streptomycin, fetal bovine serum, and protein A-agarose were purchased from Invitrogen. BFA was purchased from Sigma. Trans ^{35}S-label (>70% L-methionine; >1,000 Ci/mmol) was purchased from ICN Biomedicals.

Plasmid construction. The plasmid pTrx encoding human Trx was created by amplifying Trx cDNA with the forward (encoding *Bam*HI site and translation initiation consensus: 5′-CGGGATCCGCCACCATGGTGAAGCAGATCGAGAG-CAAG-3′) and reverse (encoding a stop codon and *Eco*RI site: 5′-CGGAATTCTTAGACTAATTCATTAATGGTGGC-3′) oligonucleotides using Platinum *pfx* DNA polymerase (Invitrogen). The PCR product was digested, isolated, and subcloned into pcDNA3 at the *Bam*HI and *Eco*RI sites. A similar method was used to create the preprolactin-myc construct by using the forward (encoding *Bam*HI site and translation initiation consensus sequence: 5′-CGGGATCCGCCACCATGG ACAGCAAAGGTTCGTCGC-3′) and reverse (encoding *Not*I site: 5′- ATAGTTTAGCGGCCGCGCAGTTGT TGTTGTAGAT-GATTCTGC-3′) oligonucleotides to amplify the bovine preprolactin gene from the plasmid pT7-Bprl. The PCR product was isolated and cloned at the *Bam*HI and *Not*I sites of pcDNA3 with a pre-existing myc epitope following the *Not*I site. The active site mutant (containing a serine instead of cysteine at residue 35) was constructed with the megaprimer method of PCR using primers T7, SP6, 5′-TAGGCCCACACCACGT-GGCTGAG-3′ and 5′-GCAAAATGATCAAGCCTTTCTTTC-3′ and pTrx as template. The assembled PCR product was subcloned into the *Bam*HI and *Eco*RI sites of pcDNA3. The dimerization mutant (containing a serine instead of cysteine at residue 73) was constructed in a similar fashion using primers T7, SP6, 5′-TTTTGACTTCACACTCTGAAGC-3′, and 5′-GCA-TGCCAACATTCCAGTTTTTTAAG-3′. All plasmids were sequenced for verification.

Cell culture. CHO, MCF-7, and HT-29 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin/streptomycin, 1% nonessential amino acids, and 2 mM L-glutamine. All cells were grown in a 37°C incubator with 5% CO_2.

Transient transfection. One day before transfection, CHO cells were trypsinized and counted. One million cells were transferred to 60-mm culture dishes and left overnight to form >95% confluent monolayers. Cells were transfected by using 3 μg of plasmid DNA and 15 μl of Lipofectamine 2000 for each 6-cm dish. Each of the components was resuspended in 450 μl of OPTI-MEM, incubated for 5 min at room temperature, mixed, and incubated for a further 23 min before the addition of 4 ml of OPTI-MEM. The DNA-Lipofectamine 2000 suspension was then added to the cells [prewashed twice in phosphate-buffered saline (PBS) to remove residual serum] and incubated at 37°C overnight. The next day, transfected cells were washed twice in PBS to remove residual DNA-Lipofectamine 2000 complexes before metabolic labeling.

Metabolic labeling with ^{35}S-methionine. CHO cells were incubated in methionine-free medium (DMEM minus methionine and cysteine, 5% fetal bovine serum, 1% Glutamax I, 1% non-essential amino acids, 1× penicillin and streptomycin) for 30 min at 37°C in 5% CO_2 and labeled with 100 μCi/ml ^{35}S-methionine for 30 min. At the end of the labeling period, the cells were washed once with chase medium (DMEM, 1× penicillin and streptomycin, 1% nonessential amino acids) and incubated for the indicated amount of time. Metabolic labeling of MCF7 and HT-29 cells was performed similarly, except the cells were starved for 1 h and labeled for 1 h with 200 μCi/ml of [^{35}S]methionine. Some cells were treated with 1 μg/ml BFA during the starvation, labeling, and chase periods.

Immunoprecipitation, SDS-PAGE, and phosphor imaging. For each dish of cells, the medium was collected and cells were lysed by the addition of 1 ml of 1× TXSWB (1% Triton X-100, 100 mM Tris·HCl, pH 8, 100 mM NaCl) in the presence of 1 mM PMSF. Both cell lysate and medium were centrifuged at 16,000 *g* for 15 min to remove debris, and the supernatants were transferred to fresh 1.5-ml tubes. Aliquots of cleared cell lysate and medium were saved for assays of lactate dehydrogenase (LDH) (see *LDH assays*). For immunoprecipitation, 200 μl of 5 × TXSWB buffer (5% Triton X-100, 500 mM Tris·HCl, pH 8, 500 mM NaCl) and 1 mM PMSF were added to the cleared medium. Either 4 μl of anti-Trx antibodies or 6 μl of anti-myc antibodies prebound to protein G beads were added per 1 ml of cell lysate or medium. The samples were mixed by inversion and incubated at 4°C for 1 h before the addition of 10 μl of a suspension of protein A-agarose beads. Samples derived from U937 and IM9 cells were precleared of endogenous antibody by overnight rotation with 10 μl of a suspension of protein A-agarose beads before immunoprecipitation. The samples were rotated at 4°C overnight, washed twice in 1× TXSWB and twice in Tris-NaCl (100 mM Tris·HCl, pH 8, 100 mM NaCl), and resuspended in 1× SDS-PAGE buffer with 500 mM 1,4-dithiothreitol (DTT). The samples were incubated at 37°C for 30 min before boiling and loaded on a 15% polyacrylamide gel. At the completion of electrophoresis, the gels were destained for 30 min, soaked in 1 M sodium salicylate for 30 min, and dried. The gels were exposed to X-ray film for viewing or a phosphor-imaging screen (Molecular Dynamics) for quantitation.

LDH assay. Assays for LDH were carried out on 5-μl samples of cell lysate and medium after the chase period to

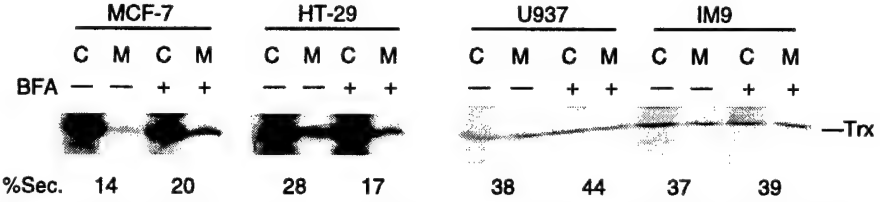

Fig. 1. Thioredoxin (Trx) is secreted from human cells via a brefeldin A (BFA)-insensitive pathway. MCF-7, HT-29, U937, and IM9 cells were incubated in methionine-free medium for 1 h, labeled with [^{35}S]methionine for 1 h, and chased in complete medium for 6 h (see METHODS for details). One dish of each cell line was treated with 1 μg/ml BFA, beginning with methionine starvation, until the end of the chase period [indicated by + over the lanes]. Cell lysates (C) and media (M) were collected and subjected to immunoprecipitation with anti-Trx antibodies. The washed immunoprecipitates were resolved by SDS-PAGE and fluorography. %Sec, percentage of Trx secreted as measured by a PhosphorImager.

assess cell lysis. The TOX7 LDH assay kit (Sigma) was used according to the manufacturer's instructions.

RESULTS

Nonclassic secretion of endogenous Trx by human cells. Trx is secreted from lymphoblastoid cells, fibroblasts, airway epithelial cells, and cells derived from tumors of breast and colon cancers (4, 28, 30, 31, 37, 38, 40). Export of Trx via a nonclassic pathway has been demonstrated only for activated lymphocytes (28). We assayed the secretion of Trx from a variety of cells expressing endogenous Trx to verify that secretion is nonclassic and to obtain a reference with which to compare secretion from heterologous cells. From 14 to 44% of Trx is secreted from MCF-7 (breast cancer), HT-29 (colon carcinoma), U937 (histiocytic lymphoma), and IM9 (multiple myeloma) cells (Fig. 1). To demonstrate that Trx is secreted from these cells via a nonclassic pathway, pulse-chase assays were repeated in the presence of BFA to block ER-Golgi transport. BFA did not block the secretion of Trx from these cells. Assays for LDH indicated that <3% of total cellular LDH was present in the medium after 4 h (data not shown), verifying that the extracellular Trx did not arise from cell lysis. Thus Trx is actively secreted from these cells via a BFA-insensitive, nonclassic pathway.

Nonclassic secretion of human Trx in a heterologous cell system. To test the role of the active site or other residues on the secretion of Trx, we searched for a mammalian cell line that secretes Trx nonclassically but lacks cross-reacting, endogenous Trx. We found that CHO cells offer several advantages for these studies. First, CHO cells do not synthesize and therefore do not secrete endogenous Trx that could interfere with our assay for secretion. Pulse-chase analysis of untransfected CHO cells with [^{35}S]methionine and subsequent immunoprecipitation with anti-Trx antibodies detected no cross-reacting protein bands corresponding to endogenous Trx in either the cell lysate or medium (Fig. 2*A*, *lanes 1* and *2*). Treatment of these cells with BFA did not alter this result (Fig. 2*A*, lanes *3* and *4*). Second, CHO cells transiently transfected with a plasmid encoding human Trx readily express an immunoreactive 12-kDa protein in the cell (Fig. 2*A*, *lane 5*). Third, Trx is also secreted into the medium (Fig. 2*A*, *lane 6*) in proportions comparable to those of cells that secrete endogenous Trx (Fig. 1). Trx is secreted via a nonclassic pathway from CHO cells because BFA did not affect this secretion (Fig. 2*A*, *lanes 7* and *8*) in contrast to its complete inhibition of prolactin secretion via the ER-Golgi pathway (Fig. 2*B*, *lanes 3* and *4*). Assays for LDH showed that <5% cell lysis occurred during the 6-h chase period (data not shown). Thus Trx is actively exported through a nonclassic pathway in CHO cells. Overall, CHO cells are a model system to study the export of Trx because these cells do not contain cross-reactive 12-kDa proteins, synthesize and export human Trx nonclassically in amounts comparable to cells that secrete endogenous Trx, and are readily transfected in contrast to the tumor cells in Fig. 1.

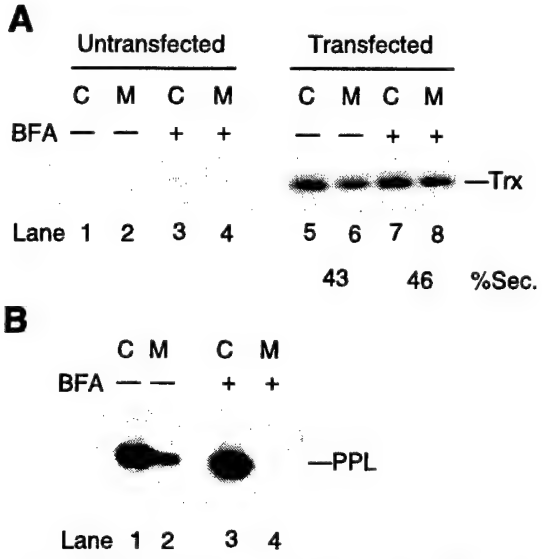

Fig. 2. Trx is secreted from transiently transfected Chinese hamster ovarian (CHO) cells through a nonclassic pathway. *A*: CHO cells were transfected with a plasmid encoding human Trx, incubated in methionine-free media for 30 min, labeled with [^{35}S]methionine for 30 min, and chased in complete media for 6 h in the absence (*lanes 1, 2, 5,* and *6*) or presence (*lanes 3, 4, 7,* and *8*) of 1 μg/ml BFA as in Fig. 1. Cell lysates (C) and media (M) were collected and subjected to immunoprecipitation with anti-Trx antibodies. %Sec, percentage of Trx secreted into the media. *B*: CHO cells were transfected with a plasmid encoding preprolactin-myc (PPL), and the experiment in *A* was performed in the absence (*lanes 1* and *2*) or presence (*lanes 3* and *4*) of BFA. The cell lysates and media were immunoprecipitated with anti-myc antibodies. %Sec, percentage of PPL secreted into the media.

Kinetics of Trx secretion by transiently transfected CHO cells. We next investigated the kinetics of Trx secretion. CHO cells transfected with Trx were labeled with [^{35}S]methionine and chased at 37°C in medium lacking serum for 2, 4, 6, and 8 h. After separation by SDS-PAGE, the protein bands were quantitated by using a PhosphorImager, and the relative secretion for each time point was plotted (Fig. 3). The secretion of Trx is a slow but steady process with up to 50% being secreted into the medium after 8 h. The increase in secretion is not due to cell lysis because an assay for LDH showed that fewer than 5% of cells lysed even after the maximum chase time of 8 h (data not shown). Because an adequate proportion of Trx is secreted into the medium after 6 h of chase, we chose to use this time point for subsequent assays of Trx secretion.

Trx secretion is altered by temperature. Facilitated protein secretion is generally a temperature-dependent process. For example, secretion of proteins via the classic ER-Golgi secretory pathway is affected by alterations in temperature (33). On the other hand, passive diffusion through a pore is not affected by a change in temperature (21). We investigated the temperature sensitivity of Trx secretion to gain insight into whether it is a facilitated or passive process. CHO cells transfected with Trx were starved, labeled at 37°C, and then incubated in serum-free chase medium at 25, 37, and 42°C for 6 h (Fig. 4). As expected, about 40% of Trx synthesized in the cells is secreted after 6 h at 37°C. Lowering the chase temperature to 25°C drastically inhibited Trx secretion. On the other hand, the secretion of Trx increased by 15% when cells were incubated at 42°C during the chase period. However, assays of LDH indicated a 15% increase in cell lysis at this temperature that accounts for much of the increase in Trx in the medium (data not shown).

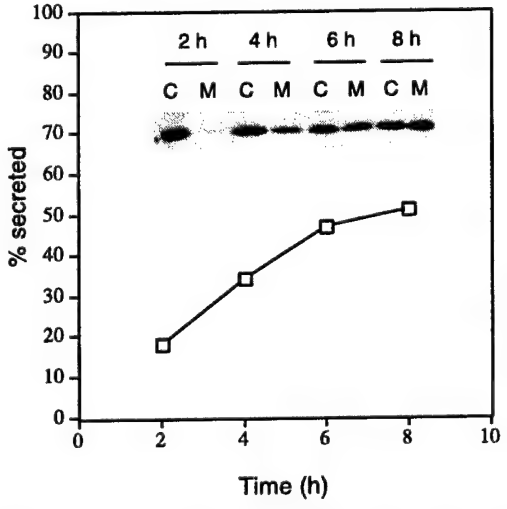

Fig. 3. Kinetics of Trx secretion by CHO cells. CHO cells transiently transfected with a plasmid encoding Trx were incubated in methionine-free media for 30 min, labeled with [^{35}S]methionine for 30 min, and chased in complete media for 2, 4, 6, or 8 h. At each time point, cell lysate (C) and medium (M) were harvested and immunoprecipitated with anti-Trx antibodies. The graph shows the percentage of Trx secreted as assessed by a phosphor imager for each chase time.

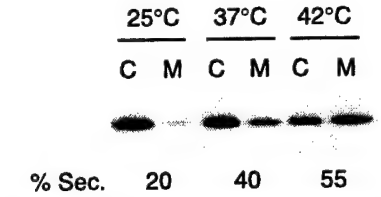

Fig. 4. Trx secretion is a temperature-dependent process. CHO cells transiently transfected with a plasmid encoding Trx were starved for methionine for 30 min, labeled with [^{35}S]methionine at 37°C for 30 min, and chased at 25, 37, or 42°C for 6 h. The cell lysate and medium were harvested and immunoprecipitated with anti-Trx antibodies as described in METHODS. %Sec, percentage of Trx secreted.

Trx secretion is affected by factors in serum. Some nonclassic secretory pathways are sensitive to factors in serum. For example, the secretion of HIV-tat and IL-1β is inversely proportional to the amount of serum in the medium (7, 29). However, the nonclassic secretion of other proteins, such as green fluorescent protein (GFP), is not affected by serum (39). We investigated whether factors present in fetal bovine serum also alter the secretion of Trx. CHO cells transfected with the Trx plasmid were starved, labeled with [^{35}S]methionine, and chased in medium containing 0, 5, 10, and 20% fetal bovine serum (Fig. 5). The proportion of secreted Trx decreased as the concentration of serum increased. A similar inhibition of secretion by serum was observed with IM9 cells secreting endogenous Trx (data not shown). Because Trx is found in serum, it is possible that Trx in bovine serum could compete with radiolabeled human Trx for immunoprecipitation. Therefore, we mixed lysates and media of transfected CHO cells with 0, 5, 10, or 20% serum before immunoprecipitation, and we found that there was no effect on the amount of labeled human Trx recovered (data not shown). Thus factors in serum decrease the secretion of Trx by CHO cells.

Secreted Trx is not associated with externalized membrane vesicles. At least one nonclassically secreted protein, galectin-3, is secreted via blebs in the plasma membrane that become externalized vesicles (20). Such a mechanism could enable the posttranslational export of fully folded cytosolic proteins from the cell. We studied whether Trx, likewise, is secreted in exter-

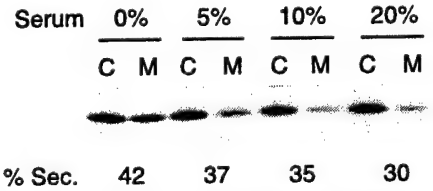

Fig. 5. The secretion of Trx is inhibited by factors in serum. CHO cells transiently transfected with a plasmid encoding Trx were incubated in methionine-free media containing 5% serum for 30 min and labeled with [^{35}S]methionine for 30 min in the same media. After being washed twice with PBS, the cells were incubated for 6 h in complete medium containing 0, 5, 10, or 20% serum. The cell lysate (C) and medium (M) were harvested and immunoprecipitated with anti-Trx antibodies. %Sec, percentage of Trx secreted.

nalized membrane vesicles via a similar pathway. CHO cells transfected with the Trx plasmid were labeled, and the medium was collected after 4 or 6 h. By examining the medium at two different times during the chase period, we would be better able to detect Trx if it is in membrane vesicles. The medium was clarified by low-speed centrifugation to remove debris and then subjected to high-speed centrifugation sufficient to pellet any vesicles (20). The supernatant and the pellet were subjected to immunoprecipitation with anti-Trx antibodies. All of the secreted Trx was in the supernatants; none of the secreted Trx was present in the pellet (Fig. 6). These data suggest that Trx is secreted freely into the medium and not in membrane vesicles from CHO cells.

N-acetyl cysteine reduces Trx secretion by downregulating Trx expression. No specific inhibitor of Trx secretion is known. Recently, it was shown that treatment with NAC resulted in the reduction of Trx secretion from ATL-2 cells (24). NAC acts as an antioxidant, and it also increases cellular glutathione levels. We investigated whether NAC actually blocks the secretory pathway used by Trx or whether the cellular level of Trx is simply decreased due to the increased availability of other antioxidants. When NAC is added during the chase period, no acute change in the secretion of Trx from CHO cells was detected (Fig. 7*A*). We also tested the effect of a longer treatment with NAC. CHO cells transiently transfected with human Trx were incubated with NAC for 16 h before assessing secretion. In these cells treated with NAC overnight, the absolute amount of Trx secreted is diminished compared with untreated cells (Fig. 7*B*). However, the level of Trx remaining in the cells treated with NAC after 6 h also is reduced so that the proportion of Trx secreted from cells treated with NAC is equivalent to that from untreated cells (Fig. 7*B*). Thus the reduction of Trx secretion by NAC is not due to a block in the Trx secretory pathway but rather from reduced cytosolic levels of Trx throughout the chase period.

Mutations in the active or regulatory homodimerization sites of human Trx do not impair secretion. Cysteine residues play key roles in the activity of Trx. The active site of human Trx consists of two cysteine residues at positions 32 and 35 relative to the NH_2 terminus. Changing either of these cysteine residues to serine renders the redox site inactive. In addition, human Trx contains cysteine at residues 62, 69, and 73. The formation of Trx homodimers that regulate the activity of the protein has been shown to depend on cysteine 73 (42).

We investigated whether the redox or regulatory status of Trx impacts its secretion. We mutated either

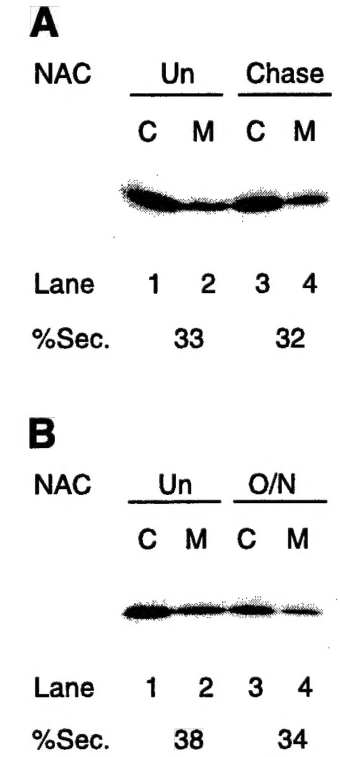

Fig. 7. *N*-acetylcysteine (NAC) lowers cytosolic levels of Trx but does not block its nonclassic secretion. *A*: CHO cells transiently expressing Trx were left untreated (Un; *lanes 1* and *2*) or treated with 10 mM NAC just during the 6-h chase period (Chase; *lanes 3* and *4*). Cell lysates (C) and media (M) were subjected to immunoprecipitation with anti-Trx antibodies. %Sec, percentage of Trx secreted. *B*: CHO cells transiently expressing Trx were left untreated (Un; *lanes 1* and *2*) or treated with 10 mM NAC beginning 16 h before and continuing throughout the labeling and chase periods (O/N; *lanes 3* and *4*). Cell lysates (C) and media (M) were subjected to immunoprecipitation with anti-Trx antibodies. %Sec, percentage of Trx secreted.

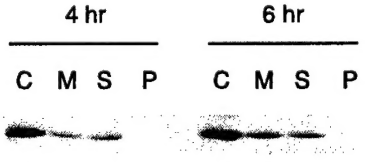

Fig. 6. Secreted Trx is not associated with externalized membrane vesicles. CHO cells transiently transfected with a plasmid encoding Trx were starved for methionine for 30 min, labeled with [^{35}S]methionine for 30 min, and chased for 4 or 6 h. At the end of the of the chase, the cell lysate (C) and medium (M) were collected and centrifuged at 2,000 *g* for 30 min, and each was divided into two equal aliquots. One aliquot of the cell lysate and medium were immunoprecipitated with anti-Trx antibodies. The second aliquot of the medium was subjected to further centrifugation at 90,000 *g* for 2 h. The supernatant (S) was collected, and the pellet (P) was resuspended in 1× TXSWB and immunoprecipitated with anti-Trx antibodies.

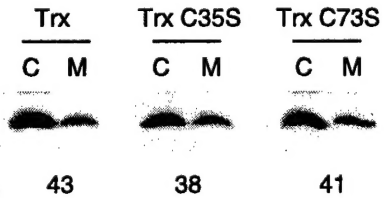

Fig. 8. Mutation of the cysteine residue at either the active site or the homodimerization site does not inhibit Trx secretion. CHO cells transiently expressing wild-type Trx (WT), a Trx-active site mutant (Trx C35S), or a Trx homodimerization site mutant (Trx C73S) were starved for methionine for 30 min, labeled with [^{35}S]methionine for 30 min, and chased for 6 h. At the end of the chase period, the cell lysate (C) and medium (M) were collected and immunoprecipitated with anti-Trx antibodies. %Sec, percentage of each Trx protein secreted.

cysteine 35 or 73 to serine to assess the effect of these disruptions on Trx secretion. Both the active site mutant (Trx C35S) and the dimerization mutant (Trx C73S) were secreted with similar efficiency to wild-type Trx (Fig. 8). Thus neither a functional redox site nor dimerization site is required for Trx secretion from CHO cells.

DISCUSSION

Several human cancer cells secrete endogenous Trx by a route other than the ER-Golgi pathway. Although secretion or surface expression of Trx from such tumor cell lines has been demonstrated previously, a nonclassic route of export has not been verified by using BFA for any cell type other than activated lymphocytes (28). Furthermore, this data establishes the proportion of Trx secreted in our assay as a standard for comparing the efficiency of secretion from heterologous CHO cells. Human Trx is secreted from CHO cells through a nonclassic pathway in amounts that compare favorably with tumor cells. The secretion of Trx is a slow but efficient process with up to 50% of the protein exported over 8 h. Secretion of Trx is reduced by low temperature and factors in serum. However, NAC does not affect secretion directly; rather, the antioxidant results in lower levels of Trx in the cytosol and medium. Using this heterologous system to study the secretion of Trx, we demonstrate that mutation of either the redox active site or the homodimerization site does not alter secretion of human Trx.

Trx is a cytosolic protein that is secreted selectively from several types of cells. Although the secretion of Trx from CHO cells is slow, it is specific and not simply due to diffusion or leakage out of the cell. First, low temperature reduces secretion of Trx. Second, we have expressed other cytosolic proteins by using transiently transfected CHO cells but did not observe any secretion into the medium (39). Therefore, the secretion of human Trx from these cells is not due to an artifact induced by liposome-mediated transfection. Third, assays of LDH show that cellular lysis is minimal and does not account for the significant proportion of Trx secreted. Thus human Trx is secreted actively via a nonclassic pathway from CHO cells.

It is interesting that the secretion of Trx from CHO cells is reduced with increasing serum concentration in the medium. Serum likewise inhibits the secretion of HIV tat protein (7). Recently, it has been shown that Trx is present in normal human serum (26). These observations raise the possibility that the nonclassic pathway by which Trx is secreted is inhibited by circulating Trx. Alternatively, other factors in serum block nonclassic secretion of Trx and other proteins such as HIV tat.

The redox status of the cytosol is maintained in part by the activity of Trx. Previously, it was demonstrated that the amount of Trx found in the media of cells treated with NAC is significantly decreased (24). These findings led to the suggestion that NAC blocks the secretion of Trx (24). Our investigations demonstrate that treatment with NAC does not have a direct effect on secretion. Instead, NAC decreases the level of Trx in the cytosol and thereby reduces the amount of Trx secreted. The proportion of Trx secreted, however, remains equivalent in the presence or absence of NAC. Furthermore, rendering the redox site of Trx inactive does not alter its secretion. Thus the redox status of the cell or Trx itself does not impact its secretion.

Proteins that are secreted generally contain a targeting signal to direct their export. In eukaryotic cells, signal sequences usually at the amino terminus of proteins mediate the targeting of proteins to the ER (5). Similarly, amino acid sequences direct secretion from bacteria from the sec-dependent or twin-arginine pathways. Therefore, it appears likely that a region of the Trx molecule functions as an export signal. We used the heterologous secretion of human Trx to study the effect of mutating the active site or a residue used in the formation of homodimers. Neither of these mutations significantly affected the secretion of Trx, suggesting that these biological activities are not involved in targeting. Little is known about targeting signals required for nonclassic protein secretion. The amino-terminal 96 amino acids of galectin-3 are necessary for its secretion (22), but the targeting signals for other nonclassically secreted proteins remain unknown. Our heterologous system of nonclassic secretion could facilitate studies of the export signal in human Trx.

Unlike other nonclassic pathways, the export route of Trx has not been identified. Several other proteins, such as IL-1β, acidic and basic fibroblast growth factor (FGF), galectins 1 and 3, and HIV-tat also appear to be secreted via nonclassic secretory pathways (6, 7, 12, 19, 20, 29). The secretion of these proteins is not attributable to cell lysis. On the basis of their response to inhibitors of secretion including serum, it appears that these proteins do not use one common pathway for nonclassic secretion. An ATP-binding cassette protein has been implicated in the secretion of IL-1β (16), a sodium-potassium ATPase has been linked to the secretion of bFGF (11), and membrane shedding results in the export of galectin 3 (20). However, the export pathway of Trx is unknown. The selective secretion of human Trx by CHO cells indicates that a nonclassic secretory pathway is present in these cells that is capable of recognizing human Trx as a substrate for export. The endogenous substrates secreted by this pathway remain to be identified.

We thank Sharmistha Ghosh for reading the manuscript and helpful discussions, Yin Chen for constructing the plasmids encoding Trx C35S and Trx C73S, Emmanuelle Wollman for providing a plasmid encoding human Trx, and David Silberstein for the generous gift of antibodies against human Trx.

This work was supported by grant DAMD17-01-1-0150 from the United States Army.

REFERENCES

1. **Arner ES and Holmgren A.** Physiological functions of thioredoxin and thioredoxin reductase. *Eur J Biochem* 267: 6102–6109, 2000.

2. **Baker A, Payne CM, Briehl MM, and Powis G.** Thioredoxin, a gene found overexpressed in human cancer, inhibits apoptosis in vitro and in vivo. *Cancer Res* 57: 5162–5167, 1997.
3. **Balcewicz-Sablinska MK, Wollman EE, Gorti R, and Silberstein DS.** Human eosinophil cytotoxicity-enhancing factor. II. Multiple forms synthesized by U937 cells and their relationship to thioredoxin/adult T cell leukemia-derived factor. *J Immunol* 147: 2170–2174, 1991.
4. **Berggren M, Gallegos A, Gasdaska JR, Gasdaska PY, Warneke J, and Powis G.** Thioredoxin and thioredoxin reductase gene expression in human tumors and cell lines, and the effects of serum stimulation and hypoxia. *Anticancer Res* 16: 3459–3466, 1996.
5. **Blobel G.** Intracellular protein topogenesis. *Proc Natl Acad Sci USA* 77: 1496–1500, 1980.
6. **Carreira CM, LaVallee TM, Tarantini F, Jackson A, Lathrop JT, Hampton B, Burgess WH, and Maciag T.** S100A13 is involved in the regulation of fibroblast growth factor-1 and p40 synaptotagmin-1 release in vitro. *J Biol Chem* 273: 22224–22231, 1998.
7. **Chang HC, Samaniego F, Nair BC, Buonaguro L, and Ensoli B.** HIV-1 Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulfate proteoglycans through its basic region. *AIDS* 11: 1421–1431, 1997.
8. **Di Trapani G, Orosco C, Perkins A, and Clarke F.** Isolation from human placental extracts of a preparation possessing 'early pregnancy factor' activity and identification of the polypeptide components. *Hum Reprod* 6: 450–457, 1991.
9. **Di Trapani G, Perkins A, and Clarke F.** Production and secretion of thioredoxin from transformed human trophoblast cells. *Mol Hum Reprod* 4: 369–375, 1998.
10. **Evan GI, Lewis GK, and Bishop JM.** Isolation of monoclonal antibodies specific for products of avian oncogene myb. *Mol Cell Biol* 4: 2843–2850, 1984.
11. **Florkiewicz RZ, Anchin J, and Baird A.** The inhibition of fibroblast growth factor-2 export by cardenolides implies a novel function for the catalytic subunit of Na^+,K^+-ATPase. *J Biol Chem* 273: 544–551, 1998.
12. **Florkiewicz RZ, Majack RA, Buechler RD, and Florkiewicz E.** Quantitative export of FGF-2 occurs through an alternative, energy-dependent, non-ER/Golgi pathway. *J Cell Physiol* 162: 388–399, 1995.
13. **Forman-Kay JD, Clore GM, Wingfield PT, and Gronenborn AM.** High-resolution three-dimensional structure of reduced recombinant human thioredoxin in solution. *Biochemistry* 30: 2685–2698, 1991.
14. **Gallegos A, Gasdaska JR, Taylor CW, Painemurrieta GD, Goodman D, Gasdaska PY, Berggren M, Briehl MM, and Powis G.** Transfection with human thioredoxin increases cell proliferation and a dominant-negative mutant thioredoxin reverses the transformed phenotype of human breast cancer cells. *Cancer Res* 56: 5765–5770, 1996.
15. **Gasdaska JR, Berggren M, and Powis G.** Cell growth stimulation by the redox protein thioredoxin occurs by a novel helper mechanism. *Cell Growth Differ* 6: 1643–1650, 1995.
16. **Hamon Y, Luciani MF, Becq F, Verrier B, Rubartelli A, and Chimini G.** Interleukin-1β secretion is impaired by inhibitors of the ATP binding cassette transporter, ABC1. *Blood* 90: 2911–2915, 1997.
17. **Hirota K, Murata M, Sachi Y, Nakamura H, Takeuchi J, Mori K, and Yodoi J.** Distinct roles of thioredoxin in the cytoplasm and in the nucleus. A two-step mechanism of redox regulation of transcription factor NF-κB. *J Biol Chem* 274: 27891–27897, 1999.
18. **Iwata S, Hori T, Sato N, Hirota K, Sasada T, Mitsui A, Hirakawa T, and Yodoi J.** Adult T cell leukemia (ATL)-derived factor/human thioredoxin prevents apoptosis of lymphoid cells induced by L-cystine and glutathione depletion: possible involvement of thiol-mediated redox regulation in apoptosis caused by pro-oxidant state. *J Immunol* 158: 3108–3117, 1997.
19. **LaVallee TM, Tarantini F, Gamble S, Carreira CM, Jackson A, and Maciag T.** Synaptotagmin-1 is required for fibroblast growth factor-1 release. *J Biol Chem* 273: 22217–22223, 1998.
20. **Mehul B and Hughes RC.** Plasma membrane targetting, vesicular budding and release of galectin 3 from the cytoplasm of mammalian cells during secretion. *J Cell Sci* 110: 1169–1178, 1997.
21. **Melchior F and Gerace L.** Mechanisms of nuclear protein import. *Curr Opin Cell Biol* 7: 310–318, 1995.
22. **Menon RP and Hughes RC.** Determinants in the N-terminal domains of galectin-3 for secretion by a novel pathway circumventing the endoplasmic reticulum-Golgi complex. *Eur J Biochem* 264: 569–576, 1999.
23. **Mitsui A, Hirakawa T, and Yodoi J.** Reactive oxygen-reducing and protein-refolding activities of adult T cell leukemia-derived factor/human thioredoxin. *Biochem Biophys Res Commun* 186: 1220–1226, 1992.
24. **Nakamura H, De Rosa SC, Yodoi J, Holmgren A, Ghezzi P, and Herzenberg LA.** Chronic elevation of plasma thioredoxin: inhibition of chemotaxis and curtailment of life expectancy in AIDS. *Proc Natl Acad Sci USA* 98: 2688–2693, 2001.
25. **Nakamura H, Herzenberg LA, Bai J, Araya S, Kondo N, Nishinaka Y, and Yodoi J.** Circulating thioredoxin suppresses lipopolysaccharide-induced neutrophil chemotaxis. *Proc Natl Acad Sci USA* 98: 15143–15148, 2001.
26. **Pekkari K, Gurunath R, Arner ES, and Holmgren A.** Truncated thioredoxin is a mitogenic cytokine for resting human peripheral blood mononuclear cells and is present in human plasma. *J Biol Chem* 275: 37474–37480, 2000.
27. **Powis G and Montfort WR.** Properties and biological activities of thioredoxins. *Annu Rev Pharmacol Toxicol* 41: 261–295, 2001.
28. **Rubartelli A, Bajetto A, Allavena G, Wollman E, and Sitia R.** Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway. *J Biol Chem* 267: 24161–24164, 1992.
29. **Rubartelli A, Cozzolino F, Talio M, and Sitia R.** A novel secretory pathway for interleukin-1β, a protein lacking a signal sequence. *EMBO J* 9: 1503–1510, 1990.
30. **Rubartelli A and Sitia R.** Interleukin 1β and thioredoxin are secreted through a novel pathway of secretion. *Biochem Soc Trans* 19: 255–259, 1991.
31. **Sahaf B, Soderberg A, Spyrou G, Barral AM, Pekkari K, Holmgren A, and Rosen A.** Thioredoxin expression and localization in human cell lines: detection of full-length and truncated species. *Exp Cell Res* 236: 181–192, 1997.
32. **Saitoh M, Nishitoh H, Fujii M, Takeda K, Tobiume K, Sawada Y, Kawabata M, Miyazono K, and Ichijo H.** Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J* 17: 2596–2606, 1998.
33. **Saraste J, Palade GE, and Farquhar MG.** Temperature-sensitive steps in the transport of secretory proteins through the Golgi complex in exocrine pancreatic cells. *Proc Natl Acad Sci USA* 83: 6425–6429, 1986.
34. **Silberstein DS, Dessein AJ, Elsas PP, Fontaine B, and David JR.** Characterization of a factor from the U937 cell line that enhances the toxicity of human eosinophils to *Schistosoma mansoni* larvae. *J Immunol* 138: 3042–3050, 1987.
35. **Silberstein DS, McDonough S, Minkoff MS, and Balcewicz-Sablinska MK.** Human eosinophil cytotoxicity-enhancing factor eosinophil-stimulating and dithiol reductase activities of biosynthetic (recombinant) species with COOH-terminal deletions. *J Biol Chem* 268: 9138–9142, 1993.
36. **Soderberg A, Sahaf B, and Rosen A.** Thioredoxin reductase, a redox-active selenoprotein, is secreted by normal and neoplastic cells: presence in human plasma. *Cancer Res* 60: 2281–2289, 2000.
37. **Tagaya Y, Maeda Y, Mitsui A, Kondo N, Matsui H, Hamuro J, Brown N, Arai K, Yokota T, Wakasugi H, and Yodoi J.** ATL-derived factor (ADF), an IL-2 receptor/Tac inducer homologous to thioredoxin; possible involvement of dithiol-reduction in the IL-2 receptor induction. *EMBO J* 8: 757–764, 1989.
38. **Tagaya Y, Okada M, Sugie K, Kasahara T, Kondo N, Hamuro J, Matsushima K, Dinarello CA, and Yodoi J.** IL-2

receptor(p55)/Tac-inducing factor. Purification and characterization of adult T cell leukemia-derived factor. *J Immunol* 140: 2614–2620, 1988.

39. **Tanudji M, Hevi S, and Chuck SL.** Improperly folded green fluorescent protein is secreted via a non-classical pathway. *J Cell Sci* 115: 3849–3857, 2002.
40. **Wakasugi H, Wakasugi N, Tursz T, Tagaya Y, and Yodoi J.** Production of an IL-1-like factor by 3B6 EBV-infected B cells [letter]. *J Immunol* 142: 2569–2570, 1989.
41. **Wakasugi N, Tagaya Y, Wakasugi H, Mitsui A, Maeda M, Yodoi J, and Tursz T.** Adult T-cell leukemia-derived factor/thioredoxin, produced by both human T-lymphotropic virus type I- and Epstein-Barr virus-transformed lymphocytes, acts as an autocrine growth factor and synergizes with interleukin 1 and interleukin 2. *Proc Natl Acad Sci USA* 87: 8282–8286, 1990.
42. **Weichsel A, Gasdaska JR, Powis G, and Montfort WR.** Crystal structures of reduced, oxidized, and mutated human thioredoxins: evidence for a regulatory homodimer. *Structure* 4: 735–751, 1996.
43. **Williams CH, Arscott LD, Muller S, Lennon BW, Ludwig ML, Wang PF, Veine DM, Becker K, and Schirmer RH.** Thioredoxin reductase two modes of catalysis have evolved. *Eur J Biochem* 267: 6110–6117, 2000.
44. **Wollman EE, d'Auriol L, Rimsky L, Shaw A, Jacquot JP, Wingfield P, Graber P, Dessarps F, Robin P, Galibert F, Bertoglio J, and Fradelizi D.** Cloning and expression of a cDNA for human thioredoxin. *J Biol Chem* 263: 15506–15512, 1988.